Neurohumoral and local controls of the electrogenic chloride secretion in rat and human epididymis with reference to the signal transduction mechanisms

A thesis submitted for the degree of

Doctor of Philosophy

by

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DECLARATION

This is to declare that the work presented in this thesis is my own and has not been submitted to this or any other institution for any degree, diploma or other qualification.

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Some of the results presented in this thesis has been reported in the following publications:

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Summary

The present study investigated the control mechanisms of transepithelial Cl^- secretion in rat epididymis, the signal transduction pathways, as well as the properties of the apical Cl^- conductance, which formed the effector in the stimulus-secretion coupling. Studies were also carried out on human epididymis to see if the results from rat epididymal tissue could be extrapolated to the human counterpart.

Using the short-circuit current (I_{SC}) technique, it was shown pharmacologically that cultured rat epididymal epithelium possessed ß1-, ß2- as well as α_1-adrenergic receptors. ß-adrenergic stimulation gave rise to increase in intracellular cAMP concentration ($[cAMP]_i$) whereas α_1-adrenergic stimulation led to Ca^{2+} release from intracellular store. Immunofluorescence studies showed that adrenergic fibres innervated the vascular and tubular smooth muscle as well as the epithelium of the rat cauda epididymidis. In addition, a novel peptide, the calcitonin gene-related peptide (CGRP), stimulated a rise in I_{SC} and $[cAMP]_i$ in a dose- and time-dependent fashion. Immunofluorescence staining of CGRP was found in the epithelial cells of the cauda epididymidis suggesting that it was synthesized *de novo* in the epithelium.

Microfluorimetric studies showed that intracellular Ca^{2+} store releasable by agonist stimulation was rapidly exchanging with the extracellular compartment. Depletion of the store activated Ca^{2+} influx across the plasma membrane. Increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was regulated by Ca^{2+} extrusion out of the cells via the Ca^{2+} ATPase and by sequestration into the intracellular store.

Using a unilateral Cl^- replacement method on short-circuit current measurement, it was found that the apical membrane of the epididymal epithelium possessed an adrenaline-activated anion conductance, which was inhibited by DPC. The selectivity sequence of the conductance was NO_3^- ~ Br^- > Cl^- > I^-> Gluconate > Isethionate. The

conductance was voltage-insensitive when the transepithelial potential difference was altered from +30 to -30 mV (apical with respect to basolateral). These properties were reminiscent of the cAMP-activated Cl^- conductance recorded by the whole-cell patch-clamp technique in epididymal (Huang et al., 1992a) and colonic tumour cells (Cliff & Frizzell, 1990). It is concluded that electrogenic Cl^- secretion in rat epididymis is subject to neural (e.g. α_1-adrenergic stimulation), humoral (e.g. ß-adrenergic stimulation) as well as local (e.g. CGRP) regulations. The stimulus-secretion coupling involves both cAMP (e.g. ß-adrenergic stimulation, CGRP) or Ca^{2+} (e.g. α_1-adrenergic stimulation, ATP) as second messengers. The effector process involves Cl^- exit across an apical DPC-sensitive Cl^- conductance.

On the other hand, studies on cultured human epididymal epithelium showed that adrenaline, forskolin and the Ca^{2+} ionophore, A23187, elicited a rise in I_{SC}, suggesting that increase in $[cAMP]_i$ or $[Ca^{2+}]_i$ stimulated transepithelial Cl^- secretion in human epididymal tissue. Moreover, stimulation with extracellular ATP elicited a transient rise in $[Ca^{2+}]_i$ in single human epididymal cells. The results demonstrated that like that in the rat, the stimulus-secretion coupling in human epididymis involved both cAMP and Ca^{2+} as second messengers. Future research work is proposed to investigate the significance of epididymal Cl^- secretion in male infertility using a gene-targeted mouse model of cystic fibrosis.

CONTENTS

Chapter I.1.

The epididymis - its structures and functions

Anatomical and histological structures of the epididymis

The epididymis forms an integral part of the male reproductive system. It is a convoluted tubular structure which is embedded in connective tissue and is enclosed within a fibrous capsule. It lies on the posterial (dorsal) aspect of the testis and is connected proximally to the rete testis via the efferent duct system and distally to the vas deferens which empties into the urethra. According to the differences in histological structures and functions, the epididymis is divided into three regions: the head (caput), the narrow body (corpus) and the tail (cauda). The area between the efferent ducts and the caput forms a region with distinctive structures and functions and is referred to as the initial segment (see fig.I.1.1.). The epididymal duct is lined by pseudostratified epithelium consisting of the principal cells as well as other cell types like the basal cells, apical cells, halo cells, clear cells and the narrow cells (Sun & Flinkinger, 1979). While the principal cells are predominant in the epithelium, the occurrence of other cell types is relatively scarce and their physiological functions remain unknown although speculations have been made on their roles as intraepithelial lymphocytes (halo cells), precursors of other cell types (narrow cells) as well as endocytotic cells (clear cells). It is on the characteristics of the principal cells that the histology of the epididymis is described.

The ultrastructures of the principal cells are characteristic of water and electrolyte transporting epithelial cells (Hinton & Turner, 1988). The surface area of the apical membrane is increased by the presence of microvilli while that of the basolateral surface is increased by extensive infoldings of the plasma membrane. The abundance of mitochondria on the basolateral aspect of the cytosol suggests that the transport processes

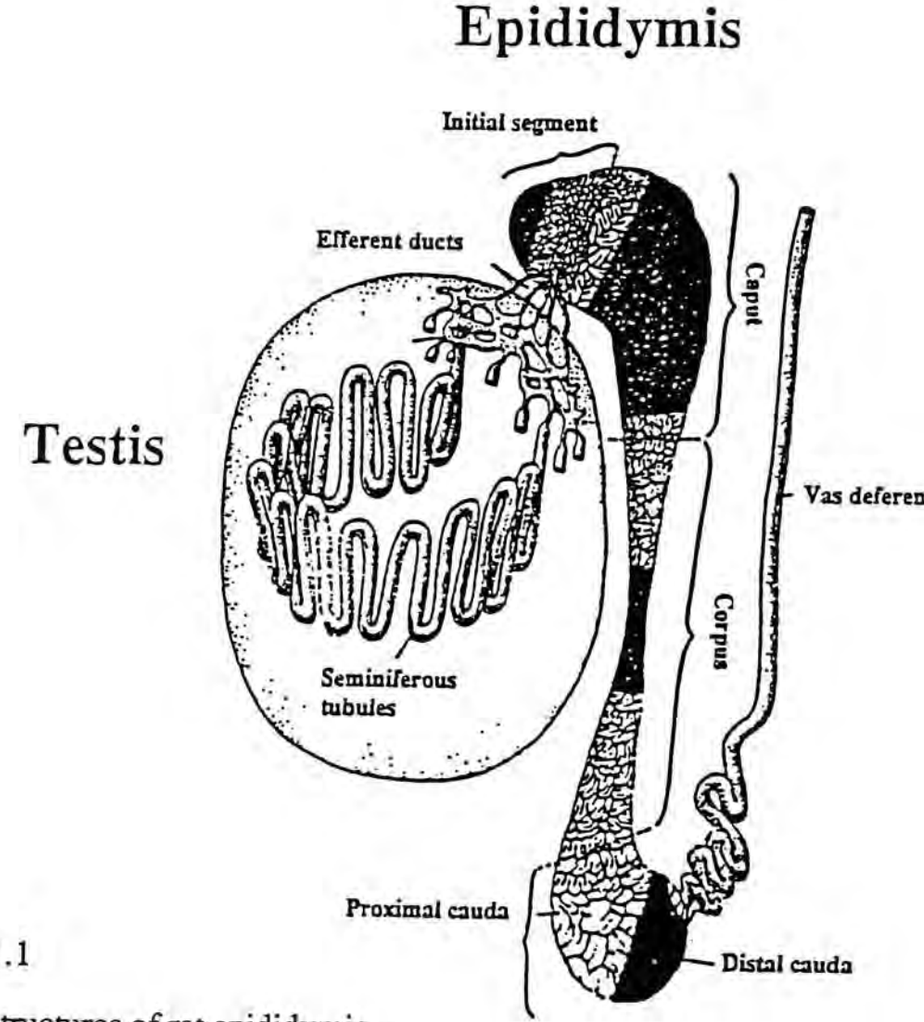


Fig.I.1.1

Basic structures of rat epididymis

are energy dependent. The histological structure of the epididymis shows progressive changes from the initial segment to the cauda epididymidis (Reid & Cleland, 1957). The diameter of the epididymal duct increases along the length of the organ and the epithelium changes from being tall columnar in the initial segment to low cuboidal in the distal region. The outer muscular layer of the epididymal duct increases from the corpus towards the vas deferens. Contraction of the tubular smooth muscle may contribute to the forward passage of spermatozoa during ejaculation.

Functions of the epididymis

It has been shown that spermatozoa aspirated in the caput region of rat epididymis are infertile while that of the caudal region have completed the final maturation changes and have acquired full fertilizing capacity (Turner, 1979). The epididymis, therefore, regulates fertility of spermatozoa by creating a unique and optimal microenvironment vital for their development (Hinton, 1980). Epididymal epithelium has been shown to secrete small organic molecules (e.g. carnitine, inositol, phosphorylcholine), steroid hormones (e.g. dihydrotestosterone) as well as various enzymes (e.g. alkaline phosphatase) into the epididymal fluid. Some of them (e.g. carnitine) are sequestered from blood to lumen while others (e.g. alkaline phosphatase) are synthesized and secreted by the epithelium (Cooper, 1986).

In addition to the secretion of organic substances, the epididymal epithelium maintains the electrolyte composition of the epididymal fluid characterized by low Na^+ and Cl^- concentrations and high K^+ concentration when compared to those in the plasma (Levine & Marsh, 1971). Specifically, Na^+ and Cl^- concentrations have been found to decrease respectively from 140 and 130 mM in the rete testis to 40 mM in the cauda epididymidis while that of K^+ rise from 10 mM in the rete testis to 40 mM in the cauda epididymidis (Jenkins et al., 1980). Most of these changes could be explained by Na^+ and

Cl^- absorption from lumen to blood and concomitant K^+ secretion in the opposite direction (Wong & Yeung, 1977, 1978). Absorption of NaCl creates an osmotic gradient for subsequent fluid absorption and the epididymis absorbs about 80% of the fluid coming from the testis (Howards et al., 1975). This leads to considerable concentration of spermatozoa in the epididymal fluid thereby facilitating their storage before ejaculation.

In addition to electrolyte and fluid absorption, recent studies using tracer $^{36}Cl^-$ fluxes have shown that the rat cauda epidiymidis secretes Cl^- ions upon stimulation with secretory agonists (Wong, 1988b). It is believed that the epididymis is capable of bidirectional transport. Fluid absorption is driven by active Na^+ transport from lumen into blood whereas fluid secretion is driven by active Cl^- transport in the opposite direction (Chapter I.2). Regulation of these processes enables fine-tuning of the fluidity or thickness of the epididymal fluid, which is crucial for the maturation and storage of spermatozoa.

Conclusion

The composition of the epididymal fluid undergoes progressive changes which begin in the caput and reach their full extent in the cauda epididymidis where the spermatozoa have acquired maturation yet remain immotile before ejaculation. The specialized microenvironment is achieved by the absorptive and secretory functions of the epithelium. In the following chapter, the cellular mechanisms of electrolyte absorption and secretion will be discussed.

Chapter I.2

Cellular mechanisms of transepithelial electrolyte transport in the epididymis and other exocrine tissues

Mechanisms of electrolyte absorption

The process of electrolyte absorption in the epididymis is similar to that in other absorptive epithelia like the frog skin (Ussing & Zerahn, 1951). Na^+ conductance resides on the apical membrane of the epithelial cells while K^+ conductance and the Na^+,K^+-ATPase are located on the basolateral membrane (Fig. I.2.1). The Na^+,K^+-ATPase extrudes 3 Na^+ out of the cells in exchange for 2 K^+ using the energy derived from the hydrolysis of ATP. K^+ diffuses out of the cells through the basolateral K^+ channels. The low intracellular Na^+ concentration generates an electrochemical gradient favouring its influx from lumen into cells across the apical membrane. The net transport of positive charges (Na^+) from lumen to blood renders the luminal side of the epithelium electronegative. The negativity provides driving force for subsequent Cl^- absorption in the same direction.

Mechanisms of electrolyte secretion

Contemporary studies on epithelial electrolyte and fluid secretion are based on the model proposed by Silva et al. (1977). Using isolated rectal gland of the dogfish, *Squalus acanthias*, it was shown that transepithelial salt secretion involves active Cl^- secretion followed by passive Na^+ diffusion through the paracellular pathway (Fig.I.2.2). Cl^- is uptaken across the basolateral membrane through a furosemide-sensitive Na^+/Cl^- carrier. The latter is driven by a low intracellular Na^+ concentration maintained by the Na^+,K^+ATPase which extrudes Na^+ out of the cells at the expense of metabolic energy derived from the hydrolysis of ATP. The net effect is the accumulation of cytosolic Cl^-

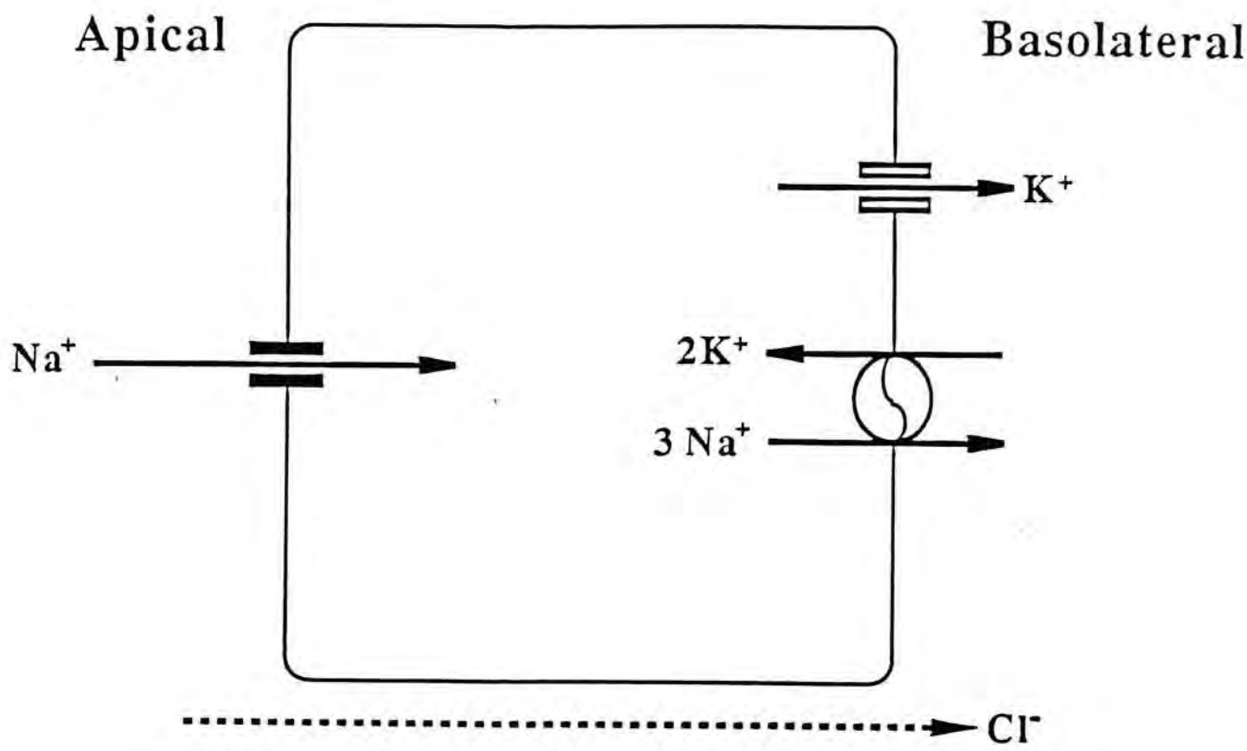


Fig.I.2.1.

Cellular mechanism of sodium absorption in the epididymis

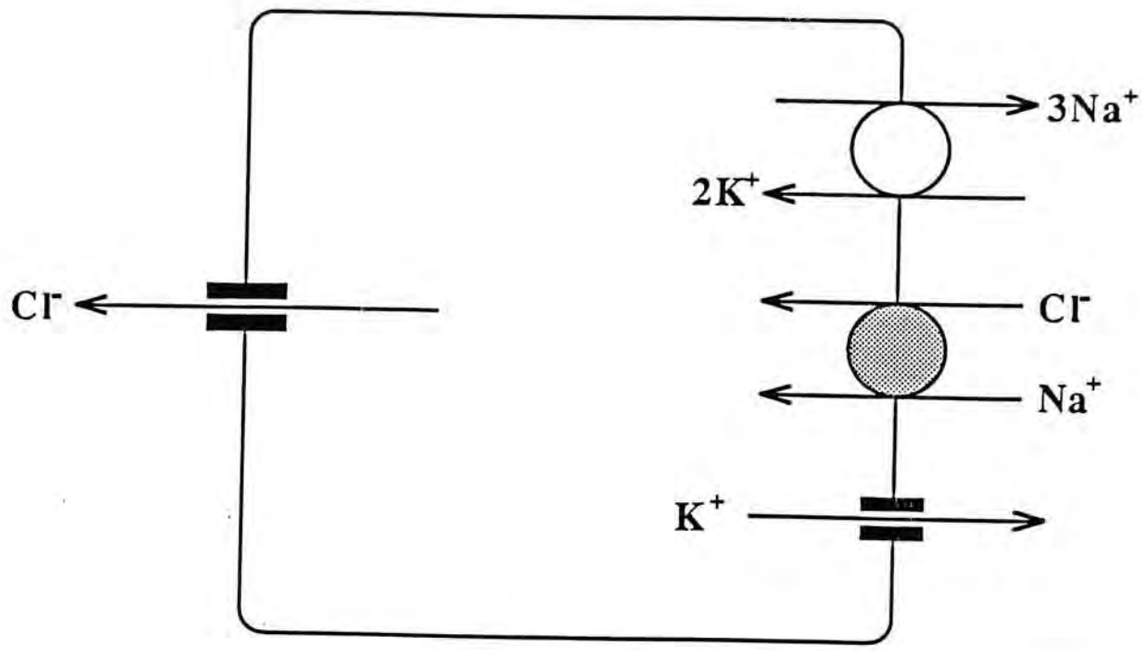


Fig.I.2.2.

Cellular mechanism of Cl^- secretion in shark rectal gland proposed by Silva et al.(1977)

above the electrochemical equilibrium. Cl^- then diffuses into the luminal compartment across the luminal membrane. The intake of K^+ through the Na^+,K^+ ATPase and the $Na^+/K^+/2Cl^-$ cotransport is balanced by its exit through K^+ channels on the basolateral membrane.

Since then, the model has been modified after the recognition that Cl^- is uptaken by a $Na^+/K^+/2Cl^-$ cotransport rather than the Na^+/Cl^- as originally proposed. The modified model seems to be energetically more efficient in terms of Cl^- uptake because 2 Cl^- ions, instead of 1, are carried into the cells during each turnover of the cotransport.

In its modified form, the model has been applied to a wide variety of other secretory epithelia including the airway epithelia (Welsh, 1987). However, more recent studies showed that in other epithelia, for instance, the rat mandibular gland (Novak & Young, 1986) and the epididymis (Wong, 1988a), the $Na^+/K^+/2Cl^-$ cotransport is not the sole driving mechanism for Cl^- secretion. In these epithelia, the cotransport is supplemented by the paired Na^+/H^+ and HCO_3^-/Cl^- exchange (Fig.I.2.3). The Na^+/H^+ exchange is driven by the low Na^+ concentration maintained by the Na^+,K^+ ATPase. The transport of H^+ out of the cells increases the intracellular HCO_3^- concentration. The latter is then extruded out of the cells via the HCO_3^-/Cl^- exchange. Therefore, the net effect of the $Na^+/K^+/2Cl^-$ cotransport, the Na^+/H^+ and the HCO_3^-/Cl^- exchanges is the accumulation of intracellular Cl^- above the electrochemical equilibrium. Agonist stimulation leads to opening of apical Cl^- channels and Cl^- diffuses across the apical membrane along the electrochemical gradient.

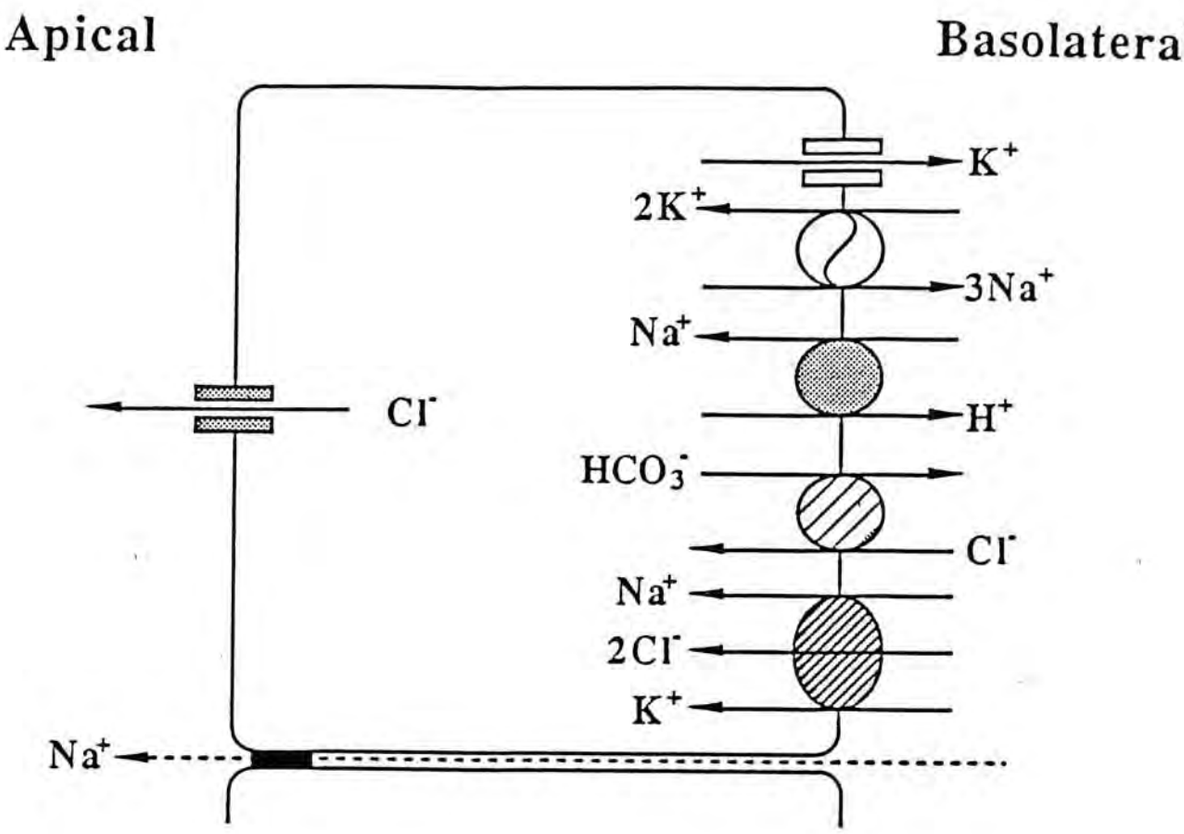


Fig.I.2.3.

Cellular mechanism of chloride secretion in the epididymis

Conclusion

The epididymis transports fluid and electrolytes in both directions. It is primarily absorptive under basal condition and becomes secretory upon agonist stimulation. The agonists activate surface receptors on the epithelium, triggering a rise in intracellular second messengers, namely, the adenosine cyclic 3':5'-monophosphate (cAMP) and Ca^{2+}. These messengers lead to opening of apical anion channels. The cAMP and Ca^{2+} signal transduction pathways will be described in the next chapter.

Chapter I.3

Signal transduction mechanisms of chloride secretion in the epididymis and other exocrine tissues

The cAMP pathway

Agonists which increase intracellular cAMP content, for instance, adrenaline (Levitzki, 1988) have been shown to stimulate Cl^- secretion in the epididymis (Wong, 1988a), the tracheal epithelium (Al-Bazzaz & Cheng, 1977), the cornea (Klyce & Wong, 1977) as well as the pancreatic ductal epithelium (Gray et al., 1988). The agonists bind onto specific receptors on cell surface and the agonist-receptor complex activates a guanine-nucleotide binding protein (G protein), which in turn causes the activation of a membrane bound enzyme adenylate cyclase (Fig.I.3.1.). The latter catalyses the conversion of ATP to cAMP (Levitzki, 1988).

It is believed that cAMP stimulates Cl^- secretion via a cAMP dependent protein kinase, the protein kinase A (PKA). cAMP binds on the regulatory site of PKA and causes its activation. Activated PKA opens apical Cl^- channels by catalysing the phosphorylation of channels (Gögelein, 1988). Cell permeant cAMP analogues have been shown to stimulate transepithelial Cl^- secretion in the epididymal epithelium (Wong, 1988a). Using the whole-cell patch-clamp technique, inclusion of the functional subunit of PKA in the interior of the cell has also been found to increase Cl^- conductance in the epididymal cells (Huang et al., 1992b).

The Ca^{2+}/phosphoinositides pathway

Knowledge about Ca^{2+}/phosphoinositides as second messengers in signal transduction can be dated back to the early fifties (Muallem, 1989). It is believed that

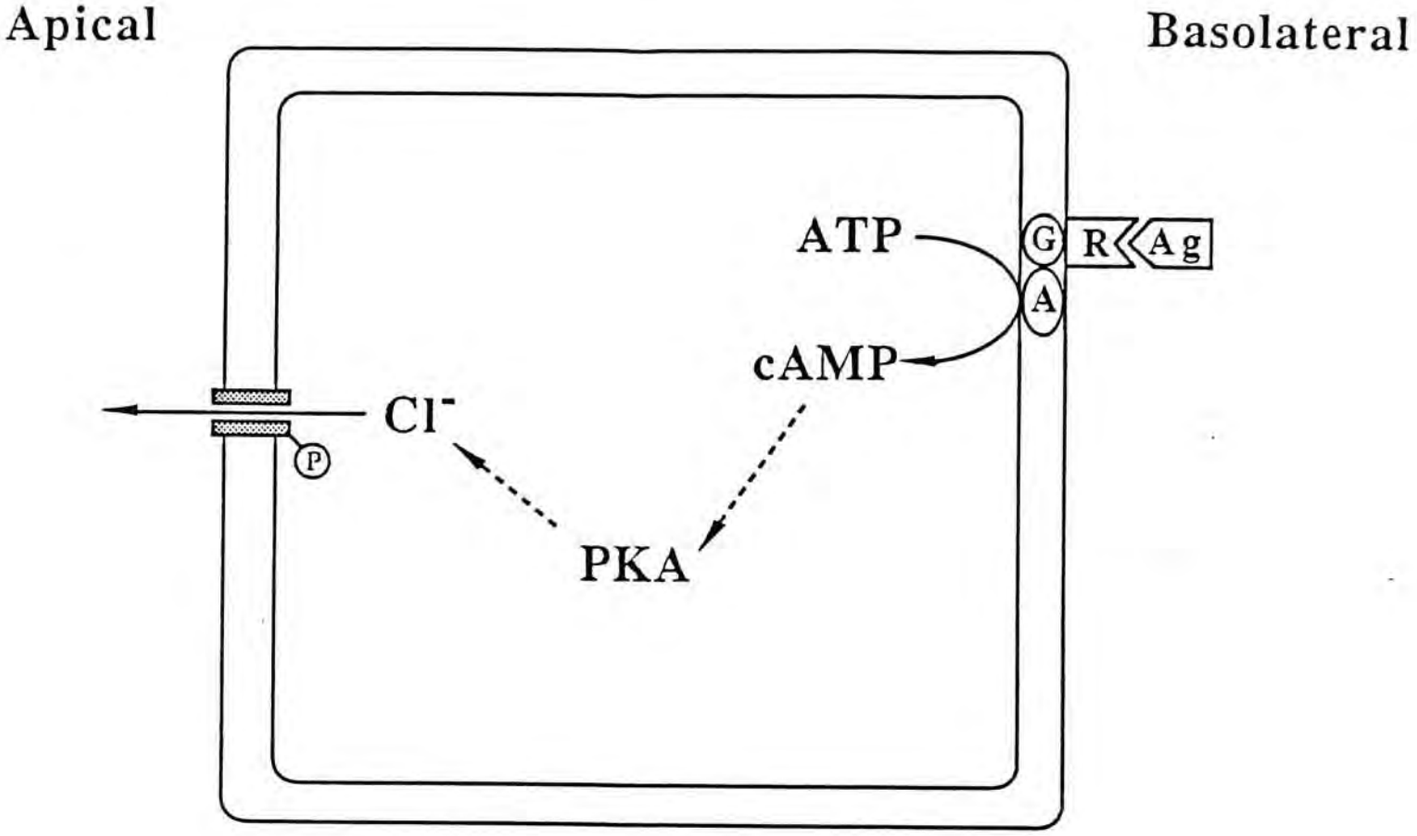


Fig.I.3.1.

The cAMP signal transduction pathway. Abbreviation: Ag: Agonist, R: Receptors, G: G protein, A: Adenylate cyclase, PKA: Protein Kinase A.

stimulation of surface receptors, for instance, α_1-adrenergic receptors (Exton, 1985) activates a membrane bound enzyme phospholipase C (PLC) via a coupling guanine-nucleotide dependent protein (G protein). PLC initiates the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), producing two intracellular messengers : Inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) (Fig.I.3.2.).

Inositol-1,4,5-trisphosphate (IP_3) and calcium

The hydrolysis of PIP_2 into IP_3 and DG forms the branching point in the signal transduction process. The hydrophilic IP_3 diffuses to the cytoplasm and binds on specific receptors on the cytoplasmic side of the intracellular Ca^{2+} store (Sharp et al., 1992). The identity of this IP_3-sensitive Ca^{2+} store is unknown, although the endoplasmic reticulum (ER) or the 'calciosome' are possible candidates (Rossier & Putney, 1991). Activation of IP_3 receptors leads to opening of Ca^{2+} channels in the store. As the free Ca^{2+} concentration in the intracellular store is greater than that in the cytoplasm as a result of active sequestration, Ca^{2+} ions diffuse from the store into the cytoplasm along the chemical gradient (Muallem, 1989), thereby increasing the cytoplasmic Ca^{2+} concentration.

In addition to mobilizing internal Ca^{2+} through IP_3, many agonists promote influx of extracellular calcium. Upon stimulation, intracellular Ca^{2+} concentration shows a rapid transient rise followed by a sustained increase (Kwan et al., 1990). The initial phase of response is attributed to Ca^{2+} release from IP_3-sensitive store whereas the sustained phase can be explained by Ca^{2+} influx across the plasma membrane (Berridge & Irvine, 1989). The mechanism by which Ca^{2+} influx is activated upon agonist stimulation has not been established.

Increase in intracellular Ca^{2+} concentration has been shown to stimulate transepithelial Cl^- secretion in the epididymal epithelium (Wong, 1988a). There are two

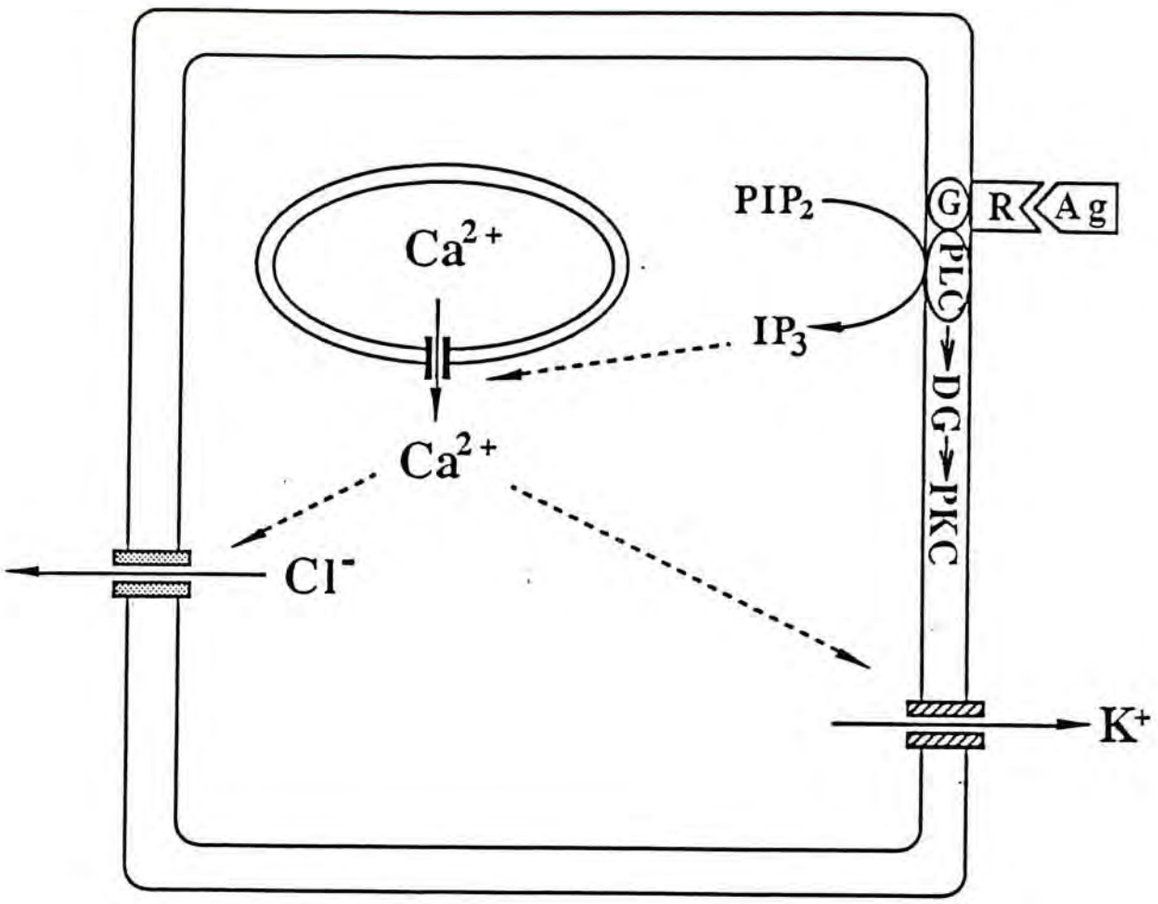


Fig.I.3.2.

The Ca^{2+}/phosphoinositides signal transduction pathway. Abbreviation: Ag: Agonist, R: Receptor, G: G protein, PLC: Phospholipase C, DG: Diacylglycerol, PKC: Protein Kinase C.

possible mechanisms. First, increase in $[Ca^{2+}]_i$ leads to opening of basolateral K^+ channels (Petersen & Maruyama, 1984). Ca^{2+} may bind directly to K^+ channels, causing conformation changes and its subsequent opening. It may also bind to an intracellular Ca^{2+} binding protein, calmodulin, and the resulting Ca^{2+}-calmodulin complex activates K^+ channels by phosphorylation through a Ca^{2+}-calmodulin kinase. K^+ efflux leads to membrane hyperpolarization thereby increasing the driving force for Cl^- exit across the apical membrane (McCann et al., 1989). Secondly, Ca^{2+} may cause direct activation of apical Cl^- channels, leading to Cl^- exit across the apical membrane along the electrochemical gradient. Ca^{2+}-activated Cl^- conductance has been demonstrated in cultured epididymal cells (Huang et al., 1992a).

Diacyglyerol and protein kinase C

Unlike IP_3, DG generated as a product of PIP_2 hydrolysis remains bound to the plasma membrane because of its hydrophobicity. DG is capable of activating a Ca^{2+} dependent protein kinase, the protein kinase C (PKC). The latter is thought to involve in the release of cellular constituents from endocrine and exocrine tissues as well as the activation of transepithelial Cl^- secretion (Nishizuka, 1986).

Conclusions

The stimulus-secretion coupling in most exocrine tissues is mediated by changes in intracellular cAMP and/or Ca^{2+} content. It has been shown that the secretory responses of salivary (Melvin et al., 1991) and pancreatic acinar cells (Osipchuk et al., 1990) are mediated by a rise in $[Ca^{2+}]_i$ whereas those of the epididymal (Wong & Huang, 1990) and the tracheal epithelium (Smith et al., 1982) are mediated by a rise in $[cAMP]_i$. However, the involvement of one signal transduction mechanism in a particular tissue does not preclude that of the other. For instance, in addition to cAMP, the Ca^{2+} ionophore A23187

has been found to elicit a rise in Cl^- secretion (Wong, 1988a). It will be interesting to find out the role of Ca^{2+} in the stimulus-secretion coupling in the epididymis.

Chapter I.4

Significance of chloride secretion by the epididymal epithelium and the objectives of the study

Introduction

The epididymis provides the specific milieu for the storage and maturation of spermatozoa (Chapter I.1). Malfunctioning of the epididymis has been associated with unexplained male infertility (Wong, 1990). On the other hand, disruption of epididymal functions can be a way to control male fertility (Hinton, 1980). This chapter describes the significance of epididymal Cl^- secretion in male fertility and the objectives of the study.

Defective fluid secretion as the cause of obstructive azoospermia

The epididymis performs both absorptive and secretory functions (Chapter I.2.). Under basal condition, the epididymis absorbs fluid and electrolytes from lumen into blood. At the same time, high-molecular-weight glycoproteins are secreted, conferring a high viscoelasticity on the epididymal fluid. These are balanced by concomitant fluid secretion from blood to lumen such that the fluidity of the luminal content can be maintained (Wong, 1986). Defective Cl^- and hence fluid secretion increase the viscosity of the luminal content. This culminates in the formation of mucus inspissations and the subsequent blockage of the epididymal duct, a clinical manifestation known as obstructive azoospermia, which is commonly seen in patients suffering from cystic fibrosis and other cases of unexplained infertility.

Epididymal obstruction in cystic fibrosis

Cystic fibrosis is the most common fatal genetic disease of the Caucasians. It is inherited in an autosomal recessive manner and it affects 1 every 2500 newborns; the

frequency of heterozygous carrier has been estimated at about 1 in 25 (Collins, 1992). It involves primarily the airway, the exocrine pancreas and the sweat gland. The clinical manifestations are chronic, recurrent pulmonary infection; pancreatic ductal obstruction by inspissations as well as abnormally high salt concentration of sweat. The primary disturbance is Cl^- impermeability on the apical membrane of the epithelial cells, giving rise to defective Cl^- secretion in airway and pancreatic duct and reduced salt absorption in sweat duct. Patch-clamp studies have shown that apical Cl^- channels are present in CF-affected tissues, but they fail to open upon cAMP stimulation (Frizzell et al., 1986). The defect in CF is due to deletion of an amino acid, phenylalanine, at the 508th position of the Cl^- channel, which is known as the cystic fibrosis transmembrane conductance regulator (CFTR) (Rich et al., 1990). Defective CFTR may contribute to Cl^- impermeability in CF-affected tissues.

The secretory mechanisms of the epididymis are, in many respects, similar to those of other exocrine tissues like the airway and the pancreatic ductal epithelium (Chapter I.2., I.3). Male CF patients who survive to adulthood are mostly infertile because of obstructive azoospermia. Many CF infants are born with the absence of vas deferens and abnormal functions of the epididymis. It is possible that defective Cl^- secretion in the epididymis leads to the formation of mucus inspissation. The resulting ductal obstruction may cause atrophy of vas deferens and epididymis.

Disruption of epididymal functions as a way to control male fertility

The microenvironment in the epididymis is important for sperm storage and maturation (Chapter I.1.). It is believed that intervention of the secretory activity of the epididymis can perturb the microenvironment thereby interfering with sperm fertility. Several compounds (e.g. methylene dimethanesulphonate, α-chlorohydrin) have been shown to interfere with sperm maturation in the epididymis (Hinton, 1980). One of the

shortcomings of these drugs as antifertility agents is the severe side-effects which are attributed to the extention of their pharmacologic actions in other tissues. Therefore, it is important to investigate the basic functions and the uniqueness of the epididymis in order to develop effective and safe male contraceptives which act selectively on the epididymis.

Objectives of the study

The present study was carried out to investigate the control mechanisms of Cl^- secretion in the epididymis. This was achieved by studying the effects of secretory agonists and antagonists on short-circuit current. The immunofluorescence technique was used to look for nerve fibres innervating the epithelium as well as local factors synthesized *de novo* in the epididymal epithelium. Intracellular Ca^{2+} measurement and biochemical assay were performed to study the signal transduction pathways involved in the stimulus-secretion coupling. To look at the final secretory process, the short-circuit current and a unilateral Cl^- replacement method were used to investigate the apical Cl^- conductance in intact epithelium. Finally, studies on human epididymis were carried out to see if the results from rat epididymis can be applied to the human counterpart, which is crucial for the study of male infertility and the development of male contraceptive agents.

Chapter II.1.

Tissue culture from the rat cauda epididymidis

Introduction

The absorptive and secretory functions of rat epididymis have been studied using isolated tubules (Wong & Yeung, 1977) and *in situ* microperfusion techniques (Wong & Yeung, 1978). Culturing epididymal cells on pervious supports allows better manipulation of the experimental conditions and enables more economical use of tissue. This chapter describes the technique of cell culture from the rat cauda epididymidis and the morphology of cultured rat epididymal cells under light microscopy.

Methods

Preparation of pervious support for cell culture

The pervious support is a membrane filter made of cellular acetate and cellulose nitrate with pore size of 0.45 μm (HA025; Nihon millipore Kogyo K.K., Japan). Silicone rings used to confine the area of the cell monolayers were prepared from Sylgard 184 silicon elastomer kit (Dow Corning Corp., U.S.A.). A double barrel cutter was pressed against the silicone plate and a silicone ring was cut out with an inner diameter of 0.6 cm. The ring was glued to the millipore filter by the 3140 RTY coating material (Dow Corning Corp., U.S.A.). The assemblies were sterilized by irradiation with ultraviolet light and were then ready for the seeding of the epididymal cells. (Fig.II.1.1.)

Procedures of cell culture

Male Sprague-Dawley rats weighing 210 to 230 g were used as the source of tissue for primary cultures. The animals were 40 day old and were sexually immature. The

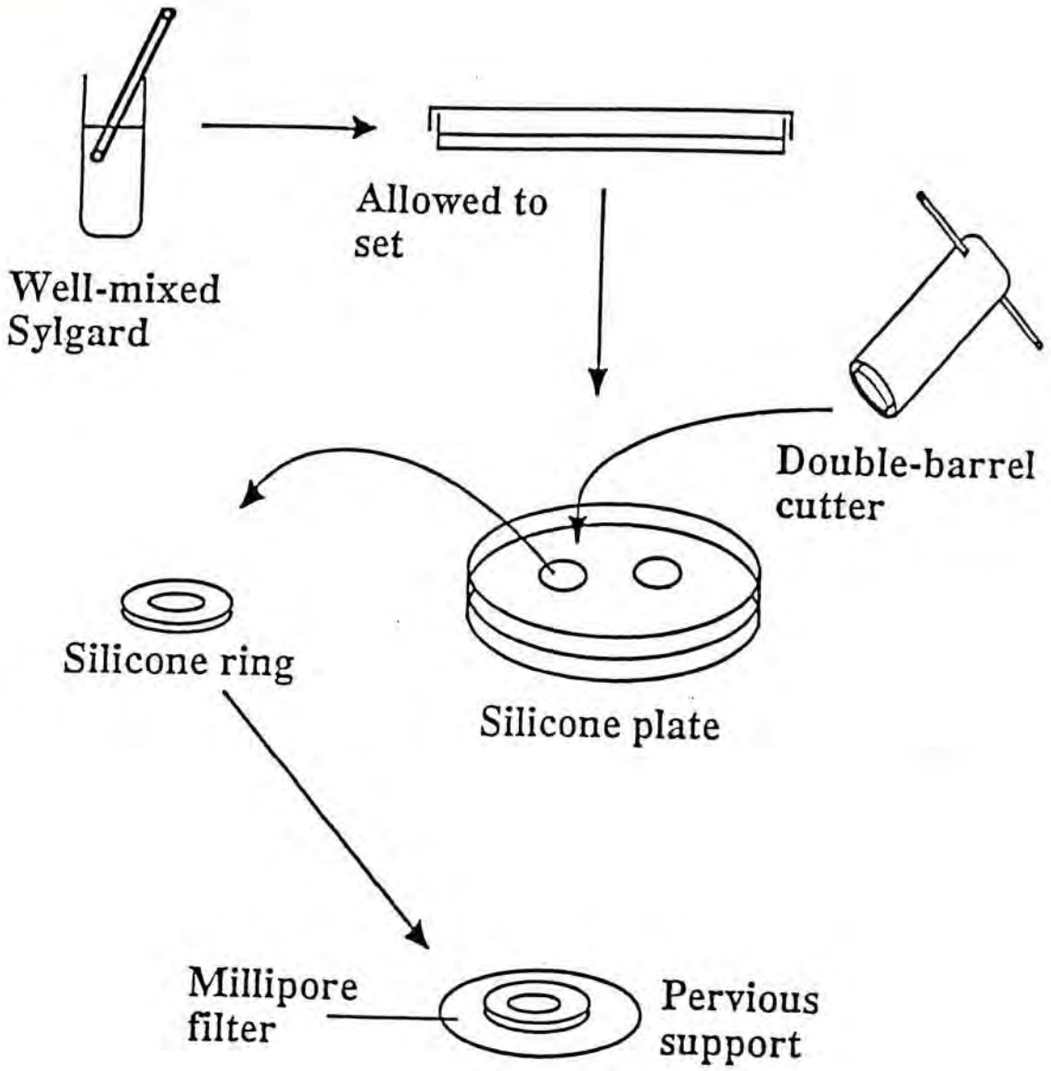


Fig.II.1.1.

Procedures for the preparation of pervious support for the short-circuit current measurement

epididymides were devoid of spermatozoa. The latter has been found to interfere with the attachment of the cells and hence their ability to form confluent monolayers.

The procedures of cell culture basically followed those described by Wong (1988a) and were shown in FigII.1.2. The rats were killed by a blow to the head followed by cervical dislocation. The lower abdomen was opened and the cauda epididymidis was dissected out. The tissue was finely chopped with scissors and then transferred to 0.25% (w/v) trypsin. The mixture was incubated for 30 minutes at 32°C with vigorous shaking (160 strokes/minutes) and then centrifuged at 800g for 5 minutes. The supernatant was discarded and the pellet of cells was resuspended in 0.1% (w/v) collagenase I. The suspension was incubated for 60 minutes at 32°C with vigorous shaking (160 strokes/minutes) and then centrifuged at 800g for 5 minutes. The cell pellet was resuspended in 4 ml of Eagle's Minimum Essential Medium (EMEM) and was incubated for 5-6 hours in 5% CO_2 at 32°C. During this period, contaminants of the primary cultures like fibroblasts and smooth muscle cells attached to the bottom of the culture flask whereas the epididymal cells remained suspended. After incubation, the cell suspension was decanted into a centrifuge tube and 0.25ml was plated onto each pervious support floating on culture medium. Cultures were incubated for 4 days at 32°C in 5% CO_2. Thereafter, the monolayers reached confluency and were ready for the short-circuit current measurement.

Histological studies of the cellular monolayers

Histological studies were carried out to look at the cellular monolayer from a cross-sectional view. The procedures of tissue fixation are tabulated in table II.1.1. The monolayer was fixed in a mixture of 2 % glutaraldehyde and 1 % formaldehyde (v/v) for 60 minutes at 4°C. It was then washed in 0.1 M phosphate buffer saline (PBS), cut into small pieces (2 x 3 mm) and postfixed for one hour in 1% osmium tetroxide at 4°C. The

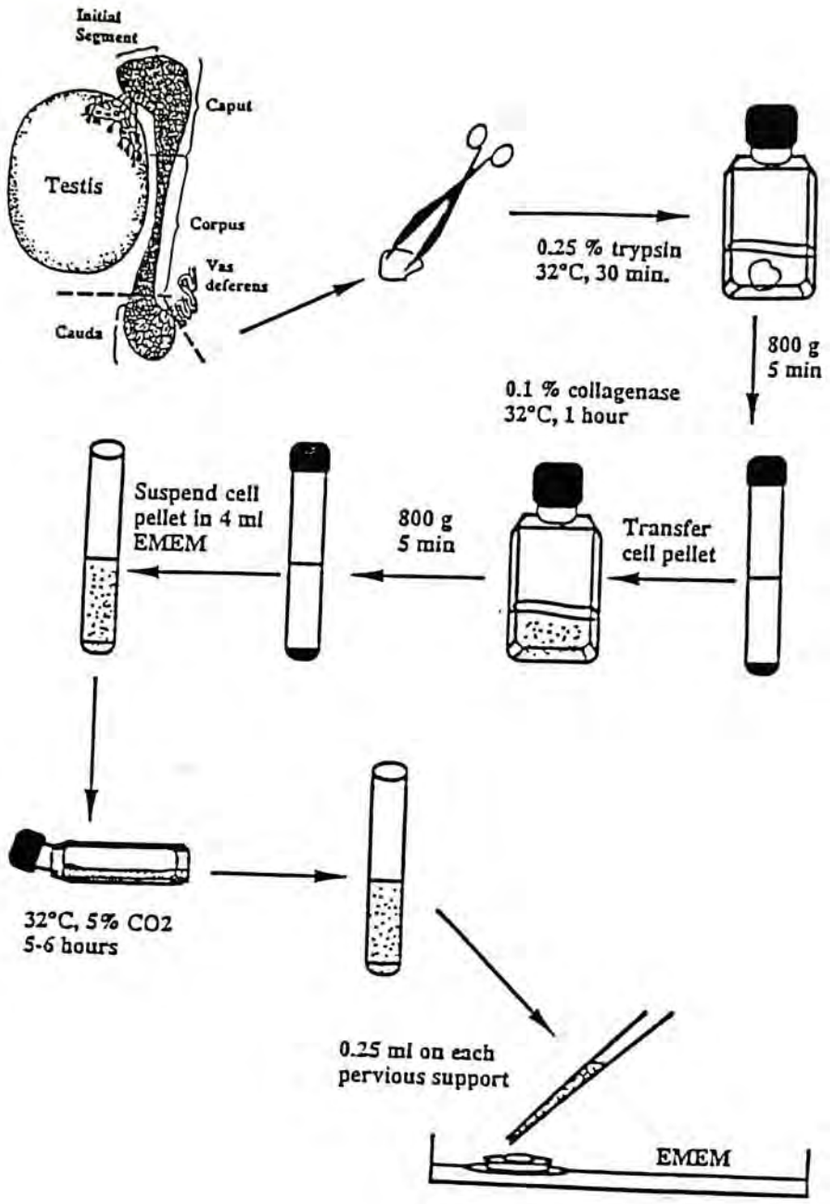


Fig.II.1.2

Procedures of cell cultures of the cauda epididymidis

small pieces were washed with Millonig's buffer (Composition see appendix I). The procedures of tissue processing were shown in table II.1.2. The small pieces of epididymal monolayers were dehydrated through a series of ethanol. To facilitate the penetration of the Spurr's Resin for embedding, the small pieces were put twice (each 15 minutes) into propylene oxide at room temperature. During the subsequent infiltration process, they were placed in a mixture of the propylene oxide and resin (propylene oxide : resin = 1:1) for two hours, followed by another mixture (propylene oxide : resin = 1:2) for further two hours. Afterwards, they were put into pure Spurr's Resin for overnight. Infiltration was then complete and the tissues were transferred to freshly prepared Spurr's Resin in a flat embedding mold. The small pieces were oriented in such a way that sections could be cut perpendicular to the epithelial sheets. The embedding molds with tissues and the Spurr's Resin in it was incubated at 63-65°C for 30 hours such that the resin could be polymerized. 0.5 μm sections were cut with a microtome (Reichert-Jung, Sweden) and mounted on glass slides. The sections were stained with 1% (w/v) toluidine blue in 1% (w/v) sodium tetraborate.

Results and discussion

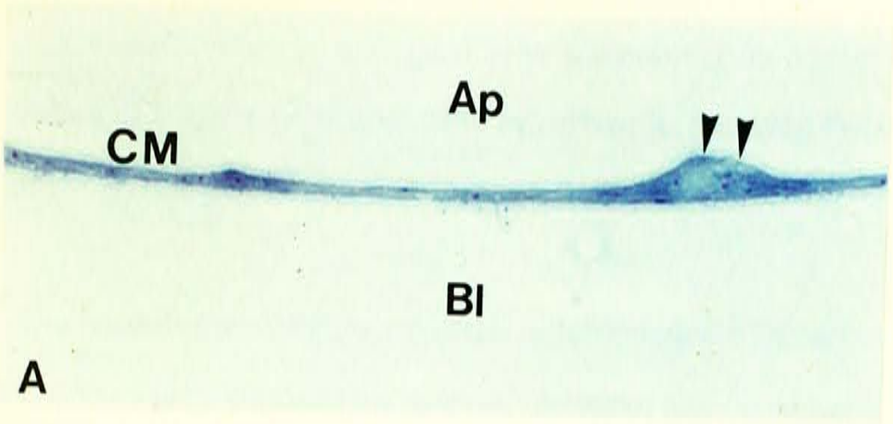
(A) of plate 1 shows a low-power photomicrograph of a cross-sectional view of the monolayer. The millipore filter has been dissolved during the processing procedures. After trypsin and collagenase digestions, the cell suspension consisted of cell clusters (double arrow heads) settling on the millipore filter. During the four day incubation, epididymal cells from the clusters spread out to form a confluent monolayer (CM). (B) is another photomicrograph at higher magnification. The epididymal cells attached on the millipore filter with their basolateral side (Bl) so that their apical side (Ap) was facing upward. The cells were cuboidal in shape and were characterized by large nuclei (large arrow) with prominent nucleoli (small arrow). Sometimes, cytoplasmic bulge could be seen to emerge from the apical cytoplasm of the cells. (C) shows a phase-contrast

Plate 1 (See text for details)

(a) A photomicrograph showing the cross-sectional view of the cellular monolayer grown in culture (x200)

(b) Similar section as (a) but at higher magnification (x1000)

(c) A phase-contrast photomicrograph of the cellular monolayer (x300)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CM
Bl
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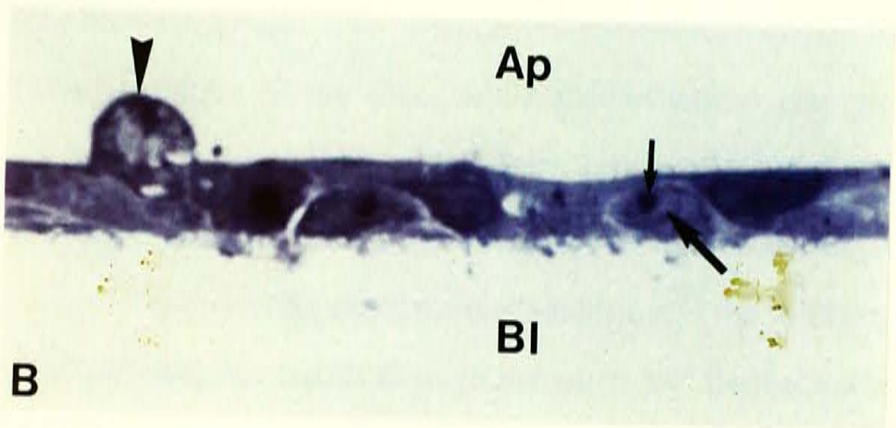
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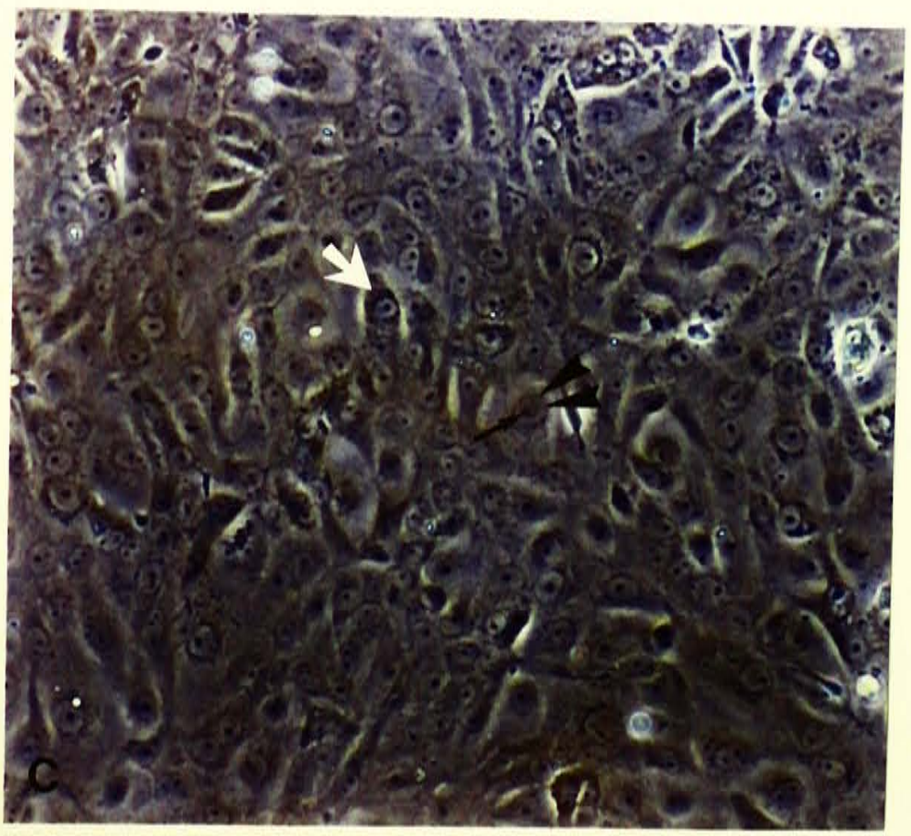

C

photomicrograph of the cellular monolayer. The epididymal cells were tightly adhered to each other so that the cell boundaries were not clearly discernible. A large and round nucleus (double arrow heads) with prominent nucleolus (dark arrow) could be seen in each epididymal cell. Cells with abundant cytoplasmic granules (white arrow) were also observed.

The reasons for using the rat cauda epididymidis as the source of tissue were two-fold. First, the functions and structures of the caput and corpus epididymidis show great differences between species. For instance, the efferent duct system in rat occupies only the very proximal margin of the caput while that in human occupies most of the caput epididymidis (Turner, 1979). Therefore, information derived from the rat caput epididymidis may not necessarily extrapolate to the human counterpart. Secondly, in most animal species, spermatozoa spend most of their time in the cauda epididymidis (about 10 days). Contrariwise, the transit through the caput and the corpus is relatively short (3-5 days) (Turner & Howards, 1977). The cauda epididymidis contains high concentration (10^9/ml) of spermatozoa which are maintained in a fully mature but quiescent state before ejaculation (Turner et al., 1977). Therefore, studies on the cauda epididymidis may be more relevant to the understanding of sperm fertility regulation.

Intact epididymal epithelium of 16 to 28 day-old rats consists of the predominant columnar cells and the sparse narrow cells. By day 28, the columnar cells develop into the principal cells and the basal cells. The narrow cells persist in the initial segment but differentiate to become the clear cells in the corpus and cauda epididymidis (Sun & Flickinger 1979). The present study could not distinguish the various cell types in the primary culture. Using electron microscopy, it has been found that cultured cells obtained from the epididymides of immature rats have ultrastructural characteristics similar to the principal cells in intact epithelium, for instance, the long microvilli and dilated vacuolar

system representing the Golgi and endoplasmic reticulum (White et al., 1982). Moreover, it has been reported that cultured epididymal cells retain their polarized nature in intact epithelium, characterized by the presence of microvilli on the apical membrane and interdigitating cytoplasmic processes between neighbouring cells some of which extend basally into the millipore filter (Yeung et al., 1989). The polarized nature of the monolayers was also evidenced by the vectorial secretion of alkaline phosphatase preferentially into the apical side of the epithelium (Cooper et al., 1989).

Cultured epididymal cells have been found to retain physiological activities *in vivo*. These include the secretion of acidic epididymal glycoprotein and the absorption of androgen binding protein (White et al., 1982); the secretion of alkaline phosphatase and N-acetylglucosaminidase, which are the enzymes normally present in the epididymal fluid (Cooper et al., 1989), as well as the ability of cellular division (Byer et al., 1985). On the other hand, Cl^- secretion by cultured epididymal epithelium have been demonstrated *in vivo* (Wong, 1988b) suggesting that the system can be used as a model for the study of epididymal anion secretion.

	FIXATION	
Glutaldehyde (2%) and paraformaldehyde (1%)	4°C	60 min
0.1 M PBS (Washing)	4°C	15 min x 3
1% osmium tetroxide	4°C	60 min
Millonig's buffer	4°C	15 min

Table II.1.1.

Procedures of tissue fixation for microtome sectioning

	Dehydration	
Ethanol (30,50,70%)	4°C	15 min each
Ethanol (70,85,95%)	Room temp.	15 min each
Absolute ethanol (I,II,III)	Room temp.	20 min x 3
	Infiltration	
Propylene oxide	Room temp.	15 min x 2
Propylene oxide and Spurr's Resin (1:1)	Room temp.	2 hours
	Embedding	
Propylene oxide and Spurr's Resin (1:2)	Room temp.	2 hours
Pure Spurr's Resin	Room temp.	Overnight
Freshly prepared pure Spurr's Resin	63-65°C	30 hours

Table II.1.2

Procedures of tissus processing for microtome sectioning

Chapter II.2.

The short-circuit current technique

Introduction

Cultured epididymal epithelium was characterized by active Cl^- transport from the basolateral to the apical side of the tissue (Chapter I.2). The rate of Cl^- transport was measured by the short-circuit current technique.

Cultured epididymal epithelium, when bathed with normal Krebs-Henseleit (K-H) solution (Appendix II), developed a transepithelial potential difference of several millivolts, with the apical side negative. It was attributed to active Cl^- secretion from the basolateral to the apical side of the cells. This created an excess of negative charges on the apical side, giving rise to the apical-negative potential difference. Na^+ were driven passively from the basolateral to the apical side along the electrical gradient.

When an external e.m.f. was applied across the epithelium to clamp the potentials on both sides equal (short-circuit), passive Na^+ transport vanished because the driving force for passive transport became zero. Under this condition, the short-circuit current measured across the epithelium was attributed to electrogenic Cl^- secretion by the epididymal cells. Agonists which stimulated Cl^- secretion gave rise to an increase in short-circuit current and agents which interfered with the secretory pathway led to a fall in current.

Experimental setup

The Ussing Chambers for short-circuit current measurement were shown in Fig.II.2.1. The pervious support (S) with the silicone ring (R) and the cultured epithelium

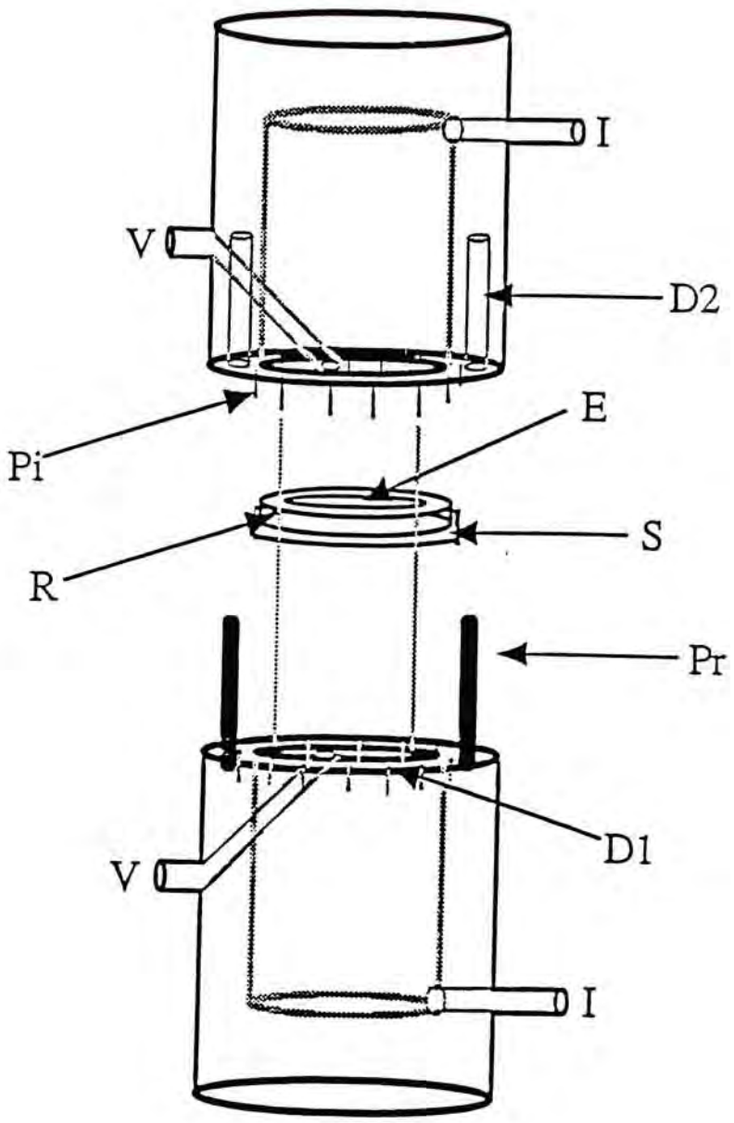


Fig.II.2.1.

The Ussing Chambers

(E) on top was put onto the upper surface of one half-chamber with small depressions (D1) arranged in a circular fashion. The other half-chamber with a ring of stainless steel pins (Pi) on it was pressed vertically onto the pervious support such that the pins were forced to pierce the silicone ring and fit into the depressions on the lower half-chamber. On the other hand, the lower half-chamber was equipped with two stainless steel projections (Pr) which could fit into the complementary depressions (D2) in the upper half-chamber. The two pairs of outlets (V and I) were connected to the agar bridges for the measurement of transepithelial potential difference and the application of external e.m.f. respectively (see below). The combination was put in a horizontal position as shown in fig.II.2.2.. The two half-chambers were pressed against each other by screws against a conical depression on one half-chamber. The opposite side of the other half chamber was fixed and the whole apparatus became water tight.

The experiment setup for short-circuit current measurement was shown in Fig.II.2.3.. Each half-chamber was connected by rubber tubings to the respective reservoir. The tissue was bathed on each side with 20 ml of bathing solution. This solution was maintained at 32°C by a water jacket surrounding the reservoirs and was bubbled with either 5% CO_2/95% O_2 or atmospheric air when HCO_3^- or HEPES was used as buffers respectively. Two narrow agar bridges A and A' (containing 0.15% (w/v) agar in 3M KCl solution) opened on either side of the epithelium. The close proximity of the agar bridges to the epithelium reduced the fluid resistance by minimizing the distance between them. The other ends of A and A' made contact with the saturated KCl- calomel electrodes through beakers containing 3M KCl. Another pair of agar bridges, B and B', opened at the far end of each chamber. The bridges were arranged as far from the

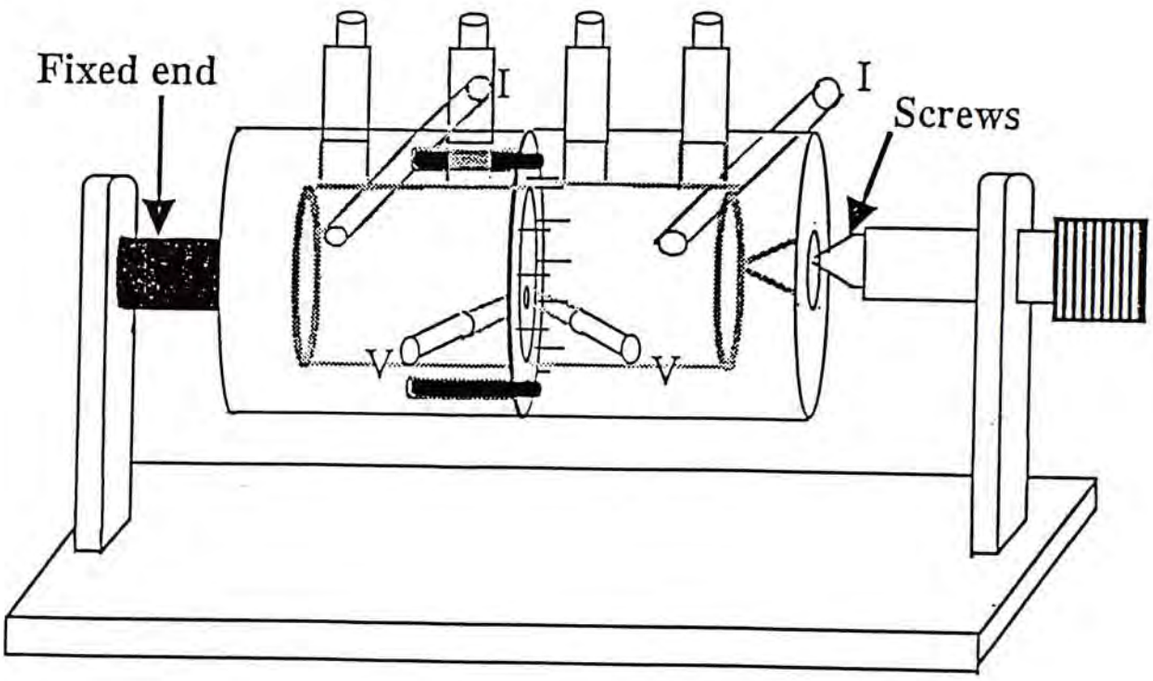


Fig.II.2.2.

The Ussing Chambers arranged in a horizontal position

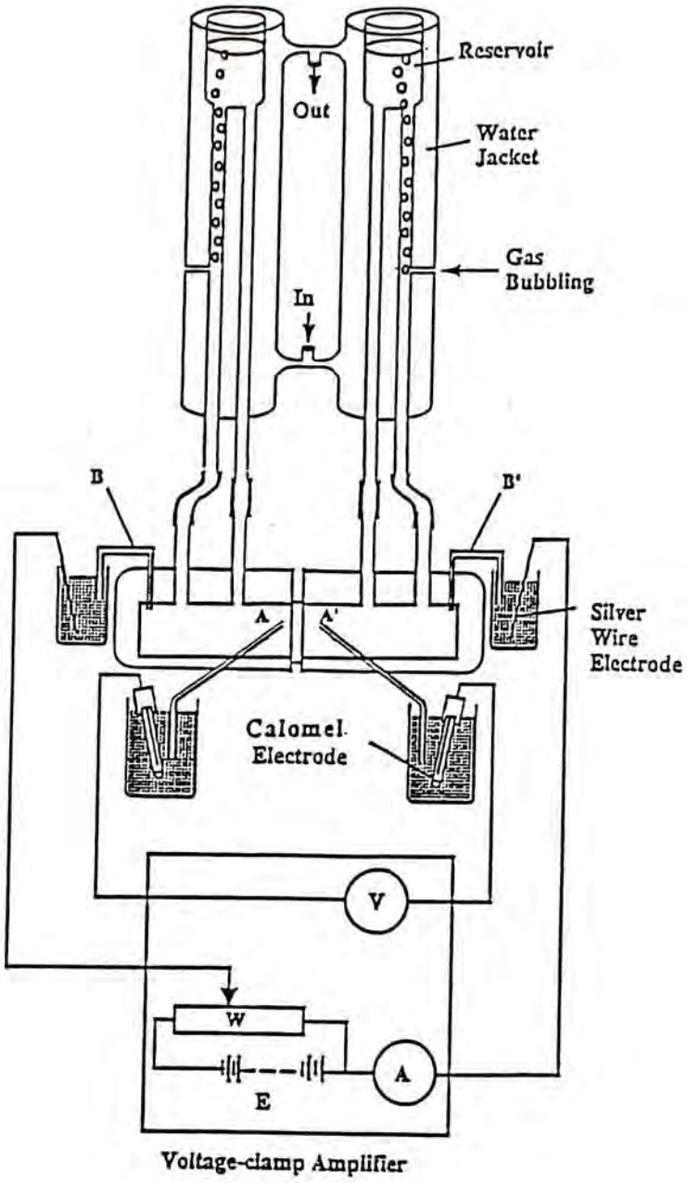


Fig.II.2.3.

Experimental setup for the short-circuit current measurment

epithelium as possible to ensure the uniformity of e.m.f. applied across the epithelium. In this way, when the external e.m.f. was applied, the potential difference between the rim and the centre of the epithelium, due to the difference in their respective distances from the bridges, could be minimized. The other ends of the bridges dipped into beakers containing 3M KCl. A pair of silver wires, immersed into these beakers, are used as electrodes through which the outer e.m.f. was applied. The calomel electrodes and the silver wire electrodes were connected to the voltage clamp amplifier (DVC-1000, World Precision Instrument, Florida, U.S.A.). The signal output from the amplifier was connected to the chart-recorder (Kipp and Zonen, Delft, the Netherlands). Drugs used in each experiment were added directly to the reservoirs leading to either the apical or the basolateral surface of the tissue.

Electrical circuitry of the voltage clamp amplifier

The voltage clamp amplifier consisted of three major components: a potentiometer (V), a D.C. electromotive force (e.m.f.) source (E) and a microameter (A). The potential difference across the epithelium was measured by the potentiometer through the calomel electrodes. Signals from the potentiometer were fed to the D.C. e.m.f. source from which an external e.m.f. was applied across the epithelium through the silver wire electrodes. The magnitude of the e.m.f. was automatically adjusted by the aid of the potential divider (W) such that both sides of the epithelium were clamped at equal potential (short-circuit). The current passing through the short-circuited epithelium was measured by the microameter. The short-circuit current was recorded on-line by the chart-recorder.

Transepithelial conductance was measured by applying intermittant voltage pulses across the epithelium and recording the corresponding current changes. The voltage pulses could be sent automatically by a built-in timer in the voltage-clamp amplifier. The timer is divided into two sections (Fig.II.2.4.). "Time A" is the period of time (20 sec) during

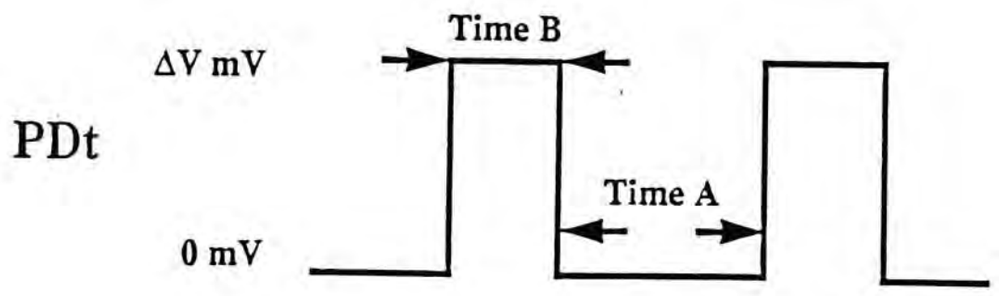


Fig.II.2.4

Time-frame of the built-in time in the voltage-clamp amplifier

which the transepithelial potential difference is clamped at zero. "Time B" is the period (1 sec) when the potential difference is clamped to some preset values. The current change (ΔI) expressed in μA due to the applied voltage pulse (ΔV) at "Time B" was read as a spike on the chart-recorder. The conductance across the epithelium was calculated according to the Ohm's Law:

$$\Delta I = \Delta V * Gt \text{ and } Gt = \Delta I / \Delta V$$

where ΔV was the clamping voltage applied (expressed in mV), ΔI was the current displacement (expressed in μA) due to ΔV, and G_t was the transepithelial conductance (expressed in mS). G_t could be calculated by dividing the current displacement (ΔI) by the voltage applied (ΔV).

Pre-use adjustment

Before each experiment, the two half chambers with a clean pervious support (a silicone ring glued onto a millipore filter) in between, the calomel electrodes as well as the silver wire electrodes, were properly connected as usual. The reservoirs were filled with physiological solutions to be used in the subsequent experiment. The junction potentials and the fluid resistance between the two calomel electrodes were nullified automatically by using the adjustment knobs on the voltage-clamp amplifier. The chambers were then disassembled and the cultured epithelium growing on pervious support was installed. The setup was reassembled and filled with physiological solutions. It was ready for the short-circuit current measurement.

At the end of each experiment, the setup was dismantled and the area of the monolayers were measured using an IBM compatible computer equipped with a digitizer (Hipad^{TM}, Houston Instruments, Austin, Texas, U.S.A.) and the software Autocad®. This was done by putting the pervious support with the epithelium on it over a gentle flame. The tissue and the millipore filter were burnt but the silicone ring was unaffected.

The ring was illuminated using a projector light and the shadow was projected onto a screen. The area enclosed by the ring (plus any surplus glue which was squeezed out when the ring was stuck on the millipore filter) was determined accurately with the digitizer. This was the actual area on which the epididymal cells were grown. The short-circuit current and the conductance across the epithelium were standardized per unit area and they were expressed as μAcm^{-2} and $mScm^{-2}$ respectively.

Chapter II.3.

The immunofluorescence technique

Introduction

Immunofluorescence staining utilizes the ability of antibody to couple specifically to the corresponding antigen. To visualize sites of immunologic reactions, the antibodies can be conjugated to fluorescent compounds which can be seen under fluorescence microscopy. Before describing the staining methods, the basic principles of immunology will be reviewed in the following paragraphs.

Basic immunology

The body of higher vertebrates has developed a defense system to protect themselves from foreign substances (antigens). It produces specific antibody to bind and neutralize the antigens. The interaction is highly specific in that a particular antibody will only recognize the antigen which triggers its production.

To produce antibody for experimental use, the purified antigen of interest is injected into an animal of a species different from that of the antigen source. The animal will identify the antigen as foreign matter and produce an antibody directed specifically against it. The antibody can be isolated from serum of the recipient animal. In the following section, the immunofluorescence staining technique, will be described.

Immunofluorescence staining

Fluorescence is the light emitted by a substance (fluorochrome) when it is excited by irradiation. When radiation of high energy falls on a substance, part of the radiant energy is absorbed and the rest is "emitted" as fluorescent light. The incident energy is

greater than the emitted energy and because of the inverse relation between radiation energy and wavelength, the wavelength of fluorescent light will be longer than that of the incident or 'exciter' light. Consequently, the fluorochrome can be excited by blue radiation (short wavelength) and be observed as yellow-green (long wavelength). The most widely used fluorochrome in immuofluorescence staining is fluoroscein isothiocyanate (FITC).

Fluorescence microscopy includes three basic components. A high-pressure mercury light source supplies the radiation for fluorescence excitation. An exciter filter transmits the effective radiation of suitable wavelength (455-490 nm for FITC) and suppresses those parts of the light source which are not used for excitation. Excessive exciter light not absorbed by the fluorochrome is suppressed by a barrier filter and only the fluorescent light with a specific wavelength (520 nm) can be transmitted and observed.

The principles of immunofluorescence technique is shown in fig. II.4.1.. Unlabelled antibody (primary antibody), specific for the antigen in question, is incubated with the tissue section. The FITC conjugated secondary antibody is allowed to bind to the primary antibody of the resulting antibody-antigen complex. Specific fluorescence from the FITC-conjugated secondary antibody provides indirect evidence for the presence of specific antibody-antigen complex formed in the first step.

To test for the specificity of interaction between the primary antibody and the tissue antigen, the pre-adsorption technique is used. The antigen of interest is incubated in excess amount with the primary antibody. This aims to adsorb the primary antibody such that all the available binding sites on it become occupied by the exogenous antigen. The adsorbed primary antibody is allowed to incubate with the tissue sections as usual. The subsequent procedures are otherwise identical to normal staining experiment. The absence of immuofluorescence staining indicates that positive staining in the normal experiment is the

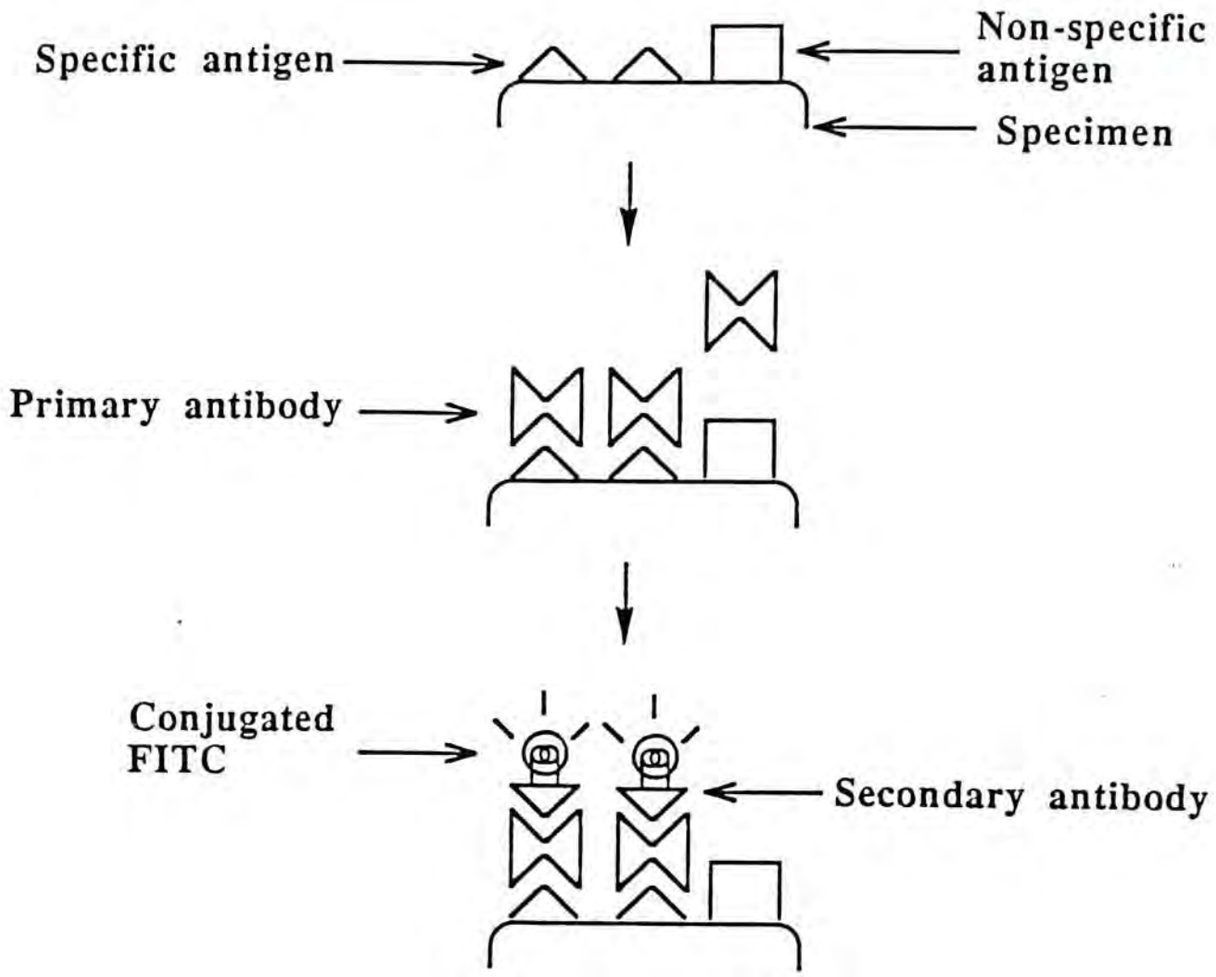


Fig.II.4.1

Diagrammatic representation showing the various components in immunofluorescence staining

result of the primary antibody-antigen complex and that the binding of the former was specific to the antigen in question.

Chapter II.4.

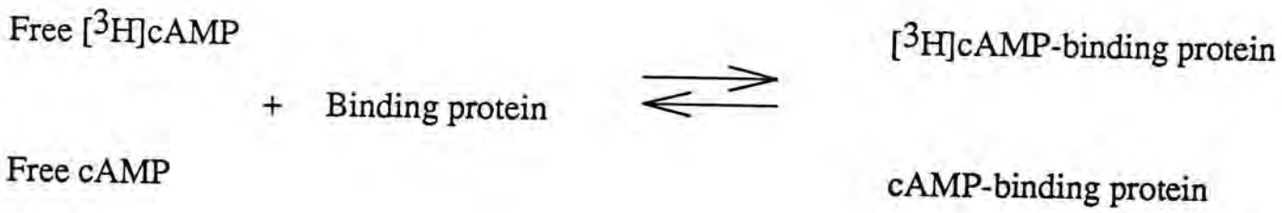
Intracellular adenosine 3':5' cyclic monophosphate (cAMP) measurement

Introduction

It has been shown that cAMP stimulates transepithelial Cl^- secretion by opening Cl^- channels on the apical membrane of secretory epithelia (Donowitz & Welsh, 1986) Measurement of intracellular cAMP concentration facilitates evaluation of the roles of this second messenger in epithelial anion transport. This chapter decribes the theory, procedures and the calculation involved in the cAMP assay.

Theory

The measurement of cAMP is based on the competition between endogenous cAMP in the sample to be assayed and a fixed quantity of the tritium labelled cAMP for binding to a limited amount of binding protein which has a high specificity and affinity for cAMP.



The greater the amount of free [^{3}H]cAMP relative to the endogenous unlabelled cAMP, the higher the proportion of binding protein which binds to the [^{3}H]cAMP. The amount of [^{3}H]cAMP-binding protein complex formed is therefore inversely related to the amount of unlabelled free cAMP present in the sample. Measurement of protein-bound radioactivity enables the amount of endogenous cAMP in the sample to be found.

The protein-bound cAMP (labelled and unlabelled) was separated from the unbound counterpart by adsorbing of the latter to activated charcoal, followed by centrifugation. The supernatant contains the protein bound cAMP whereas the residue contains the cAMP-charcoal complex. An aliquot of the supernatant is used for liquid scintillation counting. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve.

Procedures

Preparation for the serial dilutions of the standard cAMP solution

0.5 ml of the Tris-EDTA buffer solution (containing 0.05 M Tris and 4 mM EDTA, pH 7.5) was added to each of the 4 microcentrifuge tubes (numbered 1 to 4). 0.5 ml of the standard cAMP solution (0.32 μM) was added to the first tube and was vortexed. 0.5 ml of this mixture was transferred to the next tube and vortexed etc.. The steps were repeated for the remaining two tubes. Together with the original standard cAMP solution (0.32 μM), five dilutions of cAMP standards were prepared. 50 μl from each solution gave 16, 8, 4, 2 and 1 pmol per assay tube.

Assay protocol

The assay procedures were modified from those described by the assay kit (Amersham Int. Inc., Buckinghamshire, U.K.). 14 microcentrifuge tubes for standard curve and additional tubes for unknowns were numbered (see table II.3.). 150 μl of the Tris-EDTA buffer solution was pipetted into tube 1 and 2 for determining the blank counts (background counts). 50 μl of the same buffer was pipetted into tube 3 and 4 for determination of protein-bound radioactivity in the absence of unlabelled cAMP (see table II.3.). 50 μl of each dilution of the standard cAMP solution (starting with the lowest concentration) was added into successive pairs of tubes (no. 5 to 14). 50 μl of each unknown was added into the additional microcentrifuge tubes. Afterwards, 50 μl [^{3}H]-

labelled cAMP (0.072 μM) was added to every tube (no. 1 to 14 plus additional tubes for unknowns). Then 100 μl binding protein (provided by the kit) was also added to all the tubes except no. 1 and 2. The mixture was vortexed immediately and incubated at 4°C for 2 hours. After the incubation, 100 μl of ice-cooled activated charcoal suspension was added to each tube, vortexed for 5 seconds and then centrifuged at 10,000 g for 2.5 minutes. 200 μl supernatant was transferred into a 5 ml scintillation vial containing 4.5 ml of Aquasol-2 (NEN Research Products, U.S.A.) and 0.5 ml of distilled water. The contents were vortexed until it became clear and the radioactivity was measured in a scintillation counter (Model LS 7000, Beckman, U.S.A.).

Calculation

The counts per minute (cpm) for tube 1 and 2 were averaged to determine the background radioactivity. This was subtracted from the mean of tube 3 and 4 to yield the cpm bound to binding protein in the absence of unlabelled cAMP (C_o). The background cpm was also subtracted from the average cpm of each pair of tubes for standard curve (5 to 14) as well as from the cpm of each sample. This gives the specific cpm bound to binding protein (C_x) in the presence of standard (tube 5 to 14) or unknown amount of cAMP in the samples. To construct the standard curve, the ratio C_o/C_x for each dilution of the standard was plotted against the amount of cAMP (in pmoles). The standard curve of one of the assays was shown in fig. II.2. The result was a straight line with a y-intercept of ≈ 1.0. This was expected because when the standard contained no cAMP, C_x became equal to C_o and C_o/C_x became 1. From the C_o/C_x ratio, the amount of endogenous cAMP in the samples could be read from the standard curve.

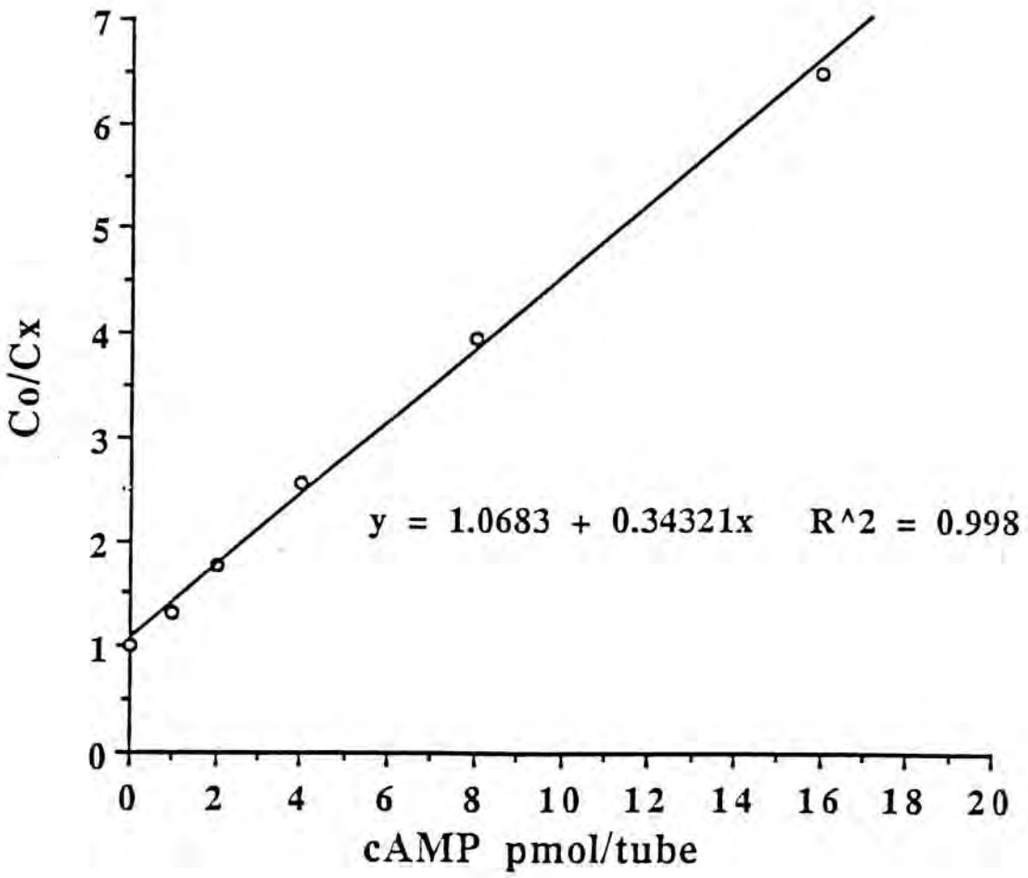


Fig.II.2.

Standard curve for the cAMP assay

Discussion

One of the features of the cAMP signal transduction pathway is that the intracellular cAMP content exhibits a transient increase upon stimulation but the cellular response (e.g. Cl^- secretion) sustains for a much longer period (Rasmussen, 1990). It is thought that the transient characteristic of the cAMP response is due to activation of phosphodiesterase which breaks down cAMP to the inactive AMP. The sustained phase of cellular reponse may be attributed to an increase in cAMP turnover rather than an actual rise in cAMP content. Therefore, the assay for cAMP could not reflect the cellular response to the added drugs/hormones. To compensate for the breakdown of cAMP, the tissue was pretreated with a phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) before stimulation with the agonists (see chapter III.1). In this way, the effects of agonists on the cellular cAMP content could be accumulated and measured.

It should be emphasized that an increase in cAMP content upon stimulation with secretory agonists does not preclude the involvement of other signal transduction mechanisms, for instance, the breakdown of membrane phosphoinositides and the subsequent increase in intracellular calcium concentration (Berridge & Irvine, 1989). The present study has measured both intracellular cAMP and Ca^{2+} concentrations. The microfluorimetric technique for Ca^{2+} measurement is described in the next chapter.

Tube no.	Tris-EDTA buffer	Serial dilution standards	Unknown sample	[^{3}H] cAMP	Binding protein	Remark
1&2	150	-	-	50	-	Background
3&4	50	-	-	50	100	0 pmol
5&6	-	50	-	50	100	1 pmol
7&8	-	50	-	50	100	2 pmol
9&10	-	50	-	50	100	4 pmol
11&12	-	50	-	50	100	8 pmol
13&14	-	50	-	50	100	16 pmol
15 etc	-	-	50	50	100	unknowns

Table II.2.

Protocol of cAMP assay. Volume is expressed in microlitres (µl).

Chapter II.5

Intracellular Ca^{2+} measurement using the microfluorimetric technique

Introduction

Intracellular Ca^{2+} is involved in various physiological activities like muscle contraction, neurotransmission as well as exocrine secretion. In the epididymis, a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) has been found to stimulate Cl^- secretion across the epithelium (Wong, 1988a). It is, therefore, pertinent to measure changes in $[Ca^{2+}]_i$ upon agonist stimulation to see if Ca^{2+} is involved in the stimulus-secretion coupling.

Theoretical background (Fig.II.5.1.)

The technique of microfluorimetry depends on a substance, the fluorochrome, which can emit fluorescence light when excited by irradiation. Each fluorochrome can be excited preferentially at a particular wavelength such that the emission intensity becomes maximum. There are a number of fluorochromes which can be used for Ca^{2+} measurement (Tsien, 1989) and the one used in the present study is called fura-2.

The fact that fura-2 can be used as a Ca^{2+} probe depends on its ability to bind Ca^{2+} due to the presence of a EGTA-backbone. However, because of the negative charges at the carboxylate endings, fura-2 cannot permeate readily through the plasma membrane. Therefore, an ester derivative of fura-2, fura-2 acetoxymethyl ester (Fura-2/AM) was used. The acetoxymethyl groups shield the negative charges at the carboxylate endings and fura-2/AM can diffuse readily into the cytosol through the plasma membrane. Because of the presence of cytoplasmic esterase, the ester bonds in fura-2/AM are hydrolysed to release the free acid.

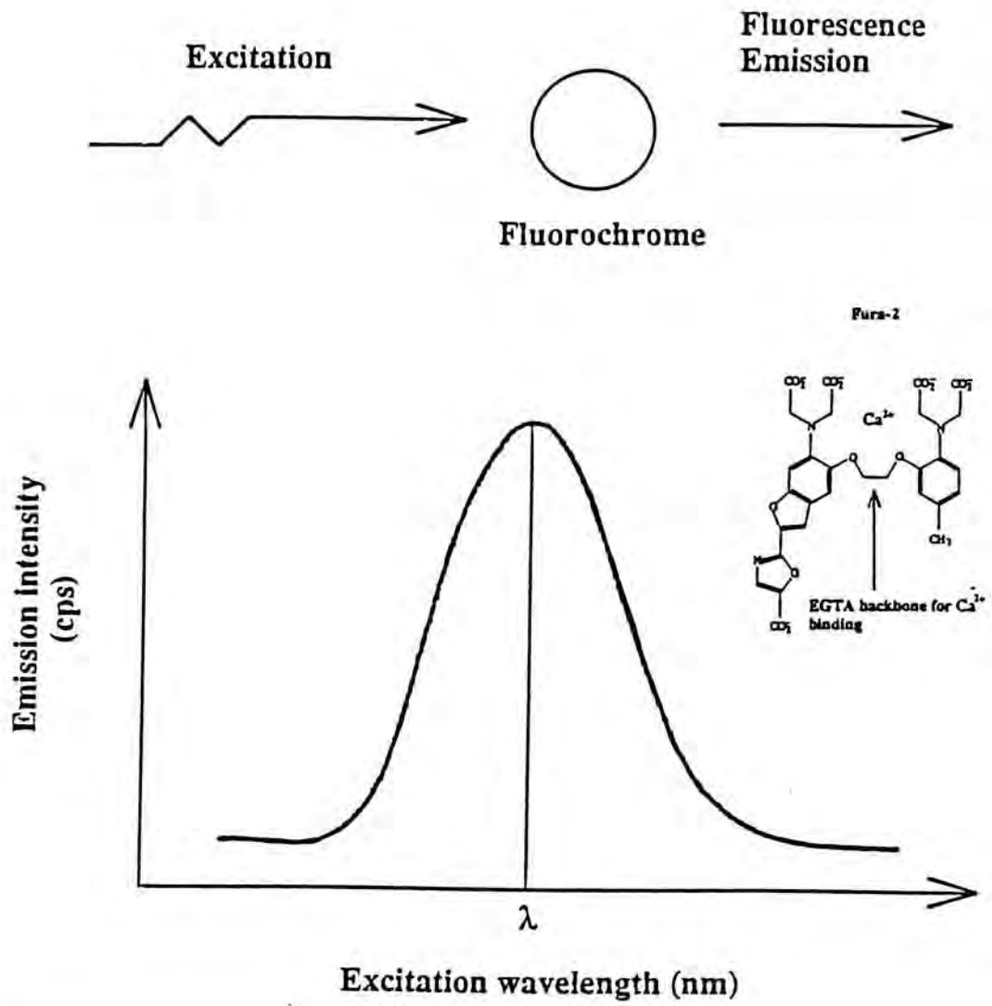


Fig.II.5.1.

The basic theory of fluorescence and the molecular structure of fura-2

The use of fura-2 as a Ca^{2+} probe is associated with the use of dual excitation wavelength. After fura-2/AM molecules have been hydrolysed, some free fura-2 molecules bind to cytoplasmic Ca^{2+} and become fura-2-Ca^{2+}. Free fura-2 without Ca^{2+} and fura-2-Ca^{2+} molecules are preferentially excited at 380 and 340 nm respectively such that their emission intensities become maximum. Upon stimulation with a Ca^{2+} mobilizing agonist, cytoplasmic Ca^{2+} concentration increases and more free fura-2 bind to Ca^{2+}. Because of the reduced amount of free fura-2, the emission intensity when excited at 380 nm decreases. On the other hand, the amount of fura-2-Ca^{2+} increases and hence the emission intensity when excited at 340 nm rises. Therefore, the ratio of emission intensities when the cell was excited at 340 nm over that at 380 nm increases. The absolute intracellular Ca^{2+} concentration can be calibrated according to this ratio such that the larger the ratio, the higher the intracellular Ca^{2+} concentration (see below).

Experimental setup (Fig.II.5.2)

The technique of microfluorimetry involves an excitation ligh source, which is a xenon arc lamp. The intensity of excitation is controlled by a shutter. A wavelength changer is used enabling the excitation wavelengths to be switched alternatively between 340 and 380 nm at high frequency. The excitation light is led into an inverted microscope operating in the epifluorescence mode. Cultured epididymal cells were grown on coverslip which was then installed in a chamber mounted on the stage of the microscope. The bathing solution is maintained thermostatically at 32°C by a steel water jacket enclosing the periphery of the chamber. Emission intensity was measured with the use of a photomulipier tube attached to the side port of the microscope. Mean fluorescence intensity for individual cells was measured within a user-defined area which covers the particular cell under study and excludes fluorescence from adjacent cells. Output from the photomultiplier tube was passed to a microcomputer for data recording and analysis. Emission intensities were collected once every 2 second and fluorescence at each excitation

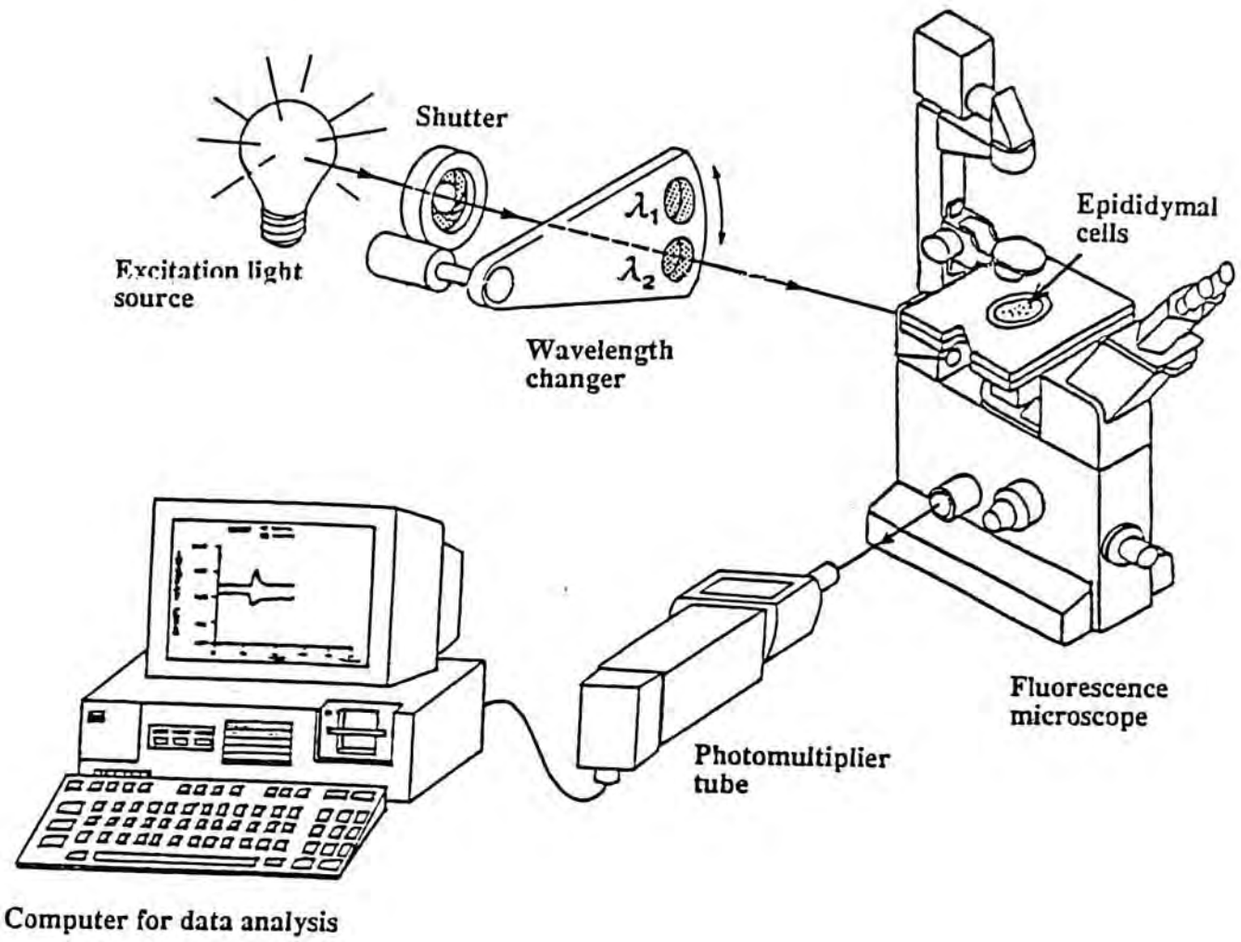


Fig.II.5.2.

Experimental setup for microfluorimetric measurement

wavelength was stored in separate file for subsequent analysis. Drugs were added to the chamber directly.

Fura-2 loading

Epididymal cells for Ca^{2+} measurement were obtained from primary cultures of the rat cauda epididymidis (see Chapter II.1.). The cells were plated onto glass coverslips and were ready for experiment after 3-4 days. During experiment, the coverslip with the epididymal cells attached to it was transferred directly from culture medium into low Ca^{2+} solution for 20 minutes (appendix III). The epididymal cells remained attached to the coverslip but were separated from each other, enabling single cells to be studied individually. The coverslip was then immersed in 6 ml normal Ca^{2+} solution (appendix III) containing 2 μM fura-2/AM. 100 μl culture medium was also added to facilitate loading. The cells were loaded at 32°C for 30 minutes with continuous stirring by a magnetic stirrer placed underneath the coverslip. After loading, the epididymal cells were bathed in normal Ca^{2+} solution for 30 to 60 minutes before fluorescence measurement was made. $[Ca^{2+}]_i$ was measured in single cells by dual excitation microfluorimetry using a SPEX DM3000 system (Spex Industries, Edison, NJ, U.S.A.).

In situ calibration of $[Ca^{2+}]_i$

At the end of each experiment, the fluorescence signals have to be calibrated into absolute value of intracellular Ca^{2+} concentration and the particular cell under study was exposed to ionomycin (40 μM) in normal Ca^{2+} solution. This allowed equilibration between intracellular and extracellular Ca^{2+} such that the binding of Ca^{2+} to intracellular Fura-2 became saturated. The fluorescence signal at 340 nm gradually increased while that at 380 nm decreased and plateau levels were attained within 20 minutes at both wavelengths. The ratio of fluorescence signal at 340 nm to that at 380 nm was defined at R_{max}. Thereafter, EGTA was added to the bathing solution to a final conncentration of 50

mM to chelate virtually all the extracellular and intracellular free Ca^{2+}. Under this circumstances, fluorescence signal at 340 nm gradually decreased while that at 380 nm increased and both signals usually levelled off within 20 minutes. The ratio of fluorescence signal at 340 nm to that at 380 nm was defined as R_{min}. About 15 % of the cells failed to exhibit this pattern of fluorescence changes upon ionomycin and EGTA addition and $[Ca^{2+}]_i$ was calibrated according to the R_{max} and R_{min} values from cells of the same batch of cultures. Finally, the cell was exposed to $MnCl_2$ (10 mM) to quench the fluorescence signal due to fura-2. The residual fluorescence at each wavelength was due to autofluorescence and fluorescence from the Ca^{2+}-insensitive forms of the dye generated by incomplete hydrolysis (Moore et al., 1990). This was subtracted from the fluorescence signal at each excitation wavelength over the entire time course of the experiment before ratioing. $[Ca^{2+}]_i$ was calculated from fluorescence ratios at saturating and zero $[Ca^{2+}]_i$ according to Grynkiewicz, Poenie & Tsien (1985),

$$[Ca^{2+}]_i = K_d \, ß \left(\frac{R - R_{min}}{R_{max} - R}\right)$$

where R indicates the ratio of fluorescence signal measured at 340 to that at 380 nm excitation, R_{max} and R_{min} have been defined previously and ß is the ratio of fluorescence signal at 380 nm excitation measured at zero (EGTA treated) $[Ca^{2+}]_i$ to that at saturated (ionomycin treated) $[Ca^{2+}]_i$. K_d is the dissociation constant of fura-2/Ca^{2+} and is taken as 224 nM.

Chapter III.1

Studies on the neural and humoral controls of electrogenic chloride secretion in rat epididymis

Summary

The present study investigated the neural and humoral controls of Cl^- secretion by rat epididymis. At low chart-speed (2 mm/min), stimulating the epididymal epithelium with noradrenaline ($ß_1$-selective agonist), salbutamol ($ß_2$-selective agonist) and adrenaline (non-selective ß-agonist) led to a transient rise in I_{SC} followed by a sustained response considerably higher than the basal I_{SC}. Pretreating the tissues with 1 μM atenolol ($ß_1$-selective antagonist) and 10 μM butoxamine ($ß_2$-selective antagonist) shifted respectively the dose-response curves of noradrenaline and salbutamol to the right. The two antagonists also shifted the dose-response curve of adrenaline to the right. The rapidly rising phase of the I_{SC} response to noradrenaline and adrenaline observed at low chart-speed consisted of a brief and transient retraction followed by a rebound increase in I_{SC}. At high chart-speed (1mm/sec), the retraction and rebound phenomenon manifested as a fast initial spike which could be blocked by phentolamine (an α-adrenergic antagonist) and BAPTA/AM (a cell permeant Ca^{2+} chelator) in a dose-dependent fashion. Noradrenaline elicited a transient rise in $[Ca^{2+}]_i$ in single epididymal cells and the response could be abolished by prior stimulation with thapsigargin, which has been shown to deplete intracellular Ca^{2+} store. Intracellular cAMP concentration also increased upon noradrenaline stimulation and the response could be abolished by a non-selective ß-antagonist, nadolol. Using the immunofluorescence technique, noradrenergic fibres were found in the tubular and vascular smooth muscle. Some fibres were seen to penetrate into the epithelium and ramify over the basal aspect of the epithelial cells. It is suggested that epididymal Cl^- secretion might be regulated by neural (noradrenaline-mediated) and

humoral (adrenaline-mediated) controls and that the stimulus-secretion coupling mechanisms involved both Ca^{2+} (α_1-mediated response) and cAMP (ß-mediated response) as intracellular second messengers.

Introduction

The rat cauda epididymidis is richly innervated by noradrenergic fibres (Kuntz & Morris, 1949). Stimulation of these fibres causes tubular smooth muscle contraction (Ventura & Pennefather, 1991). Some of the fibres have been found to penetrate the smooth muscle layer to reach the epithelium (El-Badawi & Schenk, 1967). The apposition to the epithelium suggests that sympathetic innervation may serve a secretomotor function. On the other hand, the threshold dose of adrenaline required to stimulate Cl^- secretion by the epididymis (Wong & Chan, 1988) is close to the circulating adrenaline concentration (Eisenhofer et al., 1985). It is believed that target tissues respond to nerve and circulating catecholamine stimulations through α-and ß-adrenergic receptors respectively (Bevan et al., 1980). The present study characterized the adrenoceptors involved in Cl^- secretion by cultured rat epididymal epithelium with a view to understanding the neural and humoral regulations of Cl^- secretion by rat epididymis.

Methods

Tissue culture techniques and the short-circuit current measurement (See Chapter II.1 & II.2)

Microfluorimetric technique (See Chapter II.5.)

Intracellular cAMP measurement (Also see Chapter II.4.)

Rat epididymal cells were grown on 24-well plates (Costar, Cambridge, M.A., U.S.A.). The procedures of tissue culture were basically the same as those described in chapter II.1. After collagenase digestion, the cell suspension was centrifuged for 5 minutes at 800 g. The supernatant was decanted and the pellet of cells was resuspended in 8 ml of the Eagle's Minimum Essential Medium (EMEM). 0.5 ml of cell suspension was added to each of the wells containing 0.5 ml of fresh EMEM. The epididymal cells reached confluency after 4 days in culture. During experiment, they were washed twice with Krebs-Henseleit (K-H) solution and then incubated in 0.5 ml of K-H solution containing 3

mM isobutylmethylxanthine (IBMX) for 10 minutes at 32°C (see Chapter II.4). The cells were incubated with noradrenaline for 10 minutes and the reactions were terminated by adding 10 µl of 60% (w/v) perchloric acid to each well. When the effect of a ß-antagonist, nadolol, was studied, it was added 5 minutes before noradrenaline stimulation. The content of each well was mixed and transferred to a 1.5 ml microcentrifuge tube. It was centrifuged at 10,000 g for 5 seconds and 300 µl of the supernatant was neutralized by 1 M KOH. Aliquots (50 µl) of the mixture were assayed for cAMP with the cyclic AMP kit (Chapter II.4.).

Immunofluorescence staining technique

Preparation of glass slides for cryostat sections

(i) Preparation of chrome-gelatin solution. To prepare chrome-gelatin solution (subbing solution), 2.5 g of gelatin and 0.25 g of hydrated chromium potassium sulphate ($Cr(SO_4)2.12H_2O$) were added to 500 ml of distilled water. The mixture was heated at around 70°C with continuous stirring until the solid became dissolved. The resulting solution was ready for the coating of microscopic glass slides.

(ii) Coating of glass slides. Microscopic glass slides were washed in detergent solution at 70°C for 30 min.. Afterwards, they were washed in running tap water for 2 hours and then rinsed in distilled water. The slides were dried in oven. They were then dipped in the subbing solution for a few seconds. Excess subbing solution was dripped and the "coated" slides were put in an oven at 37°C overnight. These slides were stored in dust free container and were ready for use in cryostat sectioning.

Immunofluorescence technique

Male Sprague-Dawley rats (350 g) were anaesthetized with sodium pentobarbital (60 mg/kg i.p.). The epididymides were dissected out and snap frozen in cold isopentane. 10 µm sections were cut in a cryostat at -30°C (5030 microtome, Bright, U.K.). The

sections were thaw-mounted on chrome-gelatin coated slides, air-dried and fixed with 10% formalin for 8 minutes. The staining procedures were tabulated in table III.1.1. The sections were washed three times in 0.1 M phosphate buffer saline (PBS) and incubated overnight in rabbit anti-dopamine ß hydroxylase antiserum (diluted 1:50 in 0.1 M PBS containing 0.3 % triton X-100) at 4°C. Afterwards, the sections were washed and then incubated with a secondary antibody (fluorescein isothiocyanate conjugated goat anti-rabbit IgG, diluted 1:100 in 0.1 M PBS containing 0.3% triton X-100) for one hour at room temperature. Thereafter, they were washed and mounted with glycerol (9:1 in 0.2 M Na_2HPO_4). Fluorescence was observed using a Nikon fluorescence microscope (Nikon Fluophot, Tokyo, Japan).

Drug addition in short-circuit current studies

Chemical agents were added directly to the reservoir leading to the basolateral aspect of the monolayers. To study the effects of adrenergic antagonists, the tissues were pretreated with the antagonists for 5-10 minutes before stimulation with the agonists to allow even distribution of the former within the tissues. Addition of antagonists alone did not affect the basal short-circuit current. To avoid receptor desensitization (Hausdorff et al., 1990), each monolayer was stimulated with a particular agonist only once (either in the absence or presence of antagonists).

Calculation of pK_B values

pK_B is a measure of the affinity of a competitive antagonist for its receptors. It is determined from experiments in which the tissue response to various concentrations of an agonist is inhibited by a fixed concentration of the corresponding antagonist. In the present study, the dose-response curves of noradrenaline ($ß_1$-selective agonist) or salbutamol ($ß_2$-selective agonist) were obtained in the absence and presence of atenolol ($ß_1$-antagonist) or butoxamine ($ß_2$-antagonist) respectively. The dose-ratio (DR) was calculated from the

EC_{50} in the presence of antagonist divided by that in its absence. The pK_B was calculated from DR according to the equation

$$pK_B = \log (DR-1) - \log B,$$

where B was the concentration of the antagonist expressed in M.

Although pK_B was determined from one concentration of antagonist only, it provided an estimate of the affinity of antagonist towards the receptors thereby indicating the specificity of its effects.

Loading of BAPTA acetoxymethyl ester (BAPTA/AM)

BAPTA/AM was dissolved in dimethylsulphoxide (DMSO) as 32.7 mM stock. It was added to 2 ml K-H solution in a 35 mm Petri dish to a final concentration of 50 or 100 μM. An equivolume of DMSO was added to another Petri dish as a control. The epididymal tissue was submerged in the BAPTA/AM containing K-H solution for 20 - 30 minutes in $5\%CO_2/95\%O_2$ at 32°C. Thereafter, the tissue was clamped in the Ussing Chambers as usual.

Results

Fig. III.1.1 shows the effects of ß-adrenergic agonists on I_{SC}. Noradrenaline ($ß_1$-selective agonist), salbutamol ($ß_2$-selective agonist) and adrenaline (non-selective ß-agonist) stimulated a rapid rise in I_{SC} followed by a plateau phase considerably higher than the basal I_{SC}. For quantitative studies, the I_{SC} responses to these agonists were defined as the peak increase in I_{SC} after stimulation (see below).

Fig.III.1.2 shows the dose-response relationships of noradrenaline in the absence and presence of a $ß_1$-selective antagonist, atenolol. The dose-response curves exhibited a sigmoidal profile. The threshold and EC_{50} of noradrenaline were about 1 and 300 nM respectively and the maximum I_{SC} response was attained at 1 mM. Pretreatment with

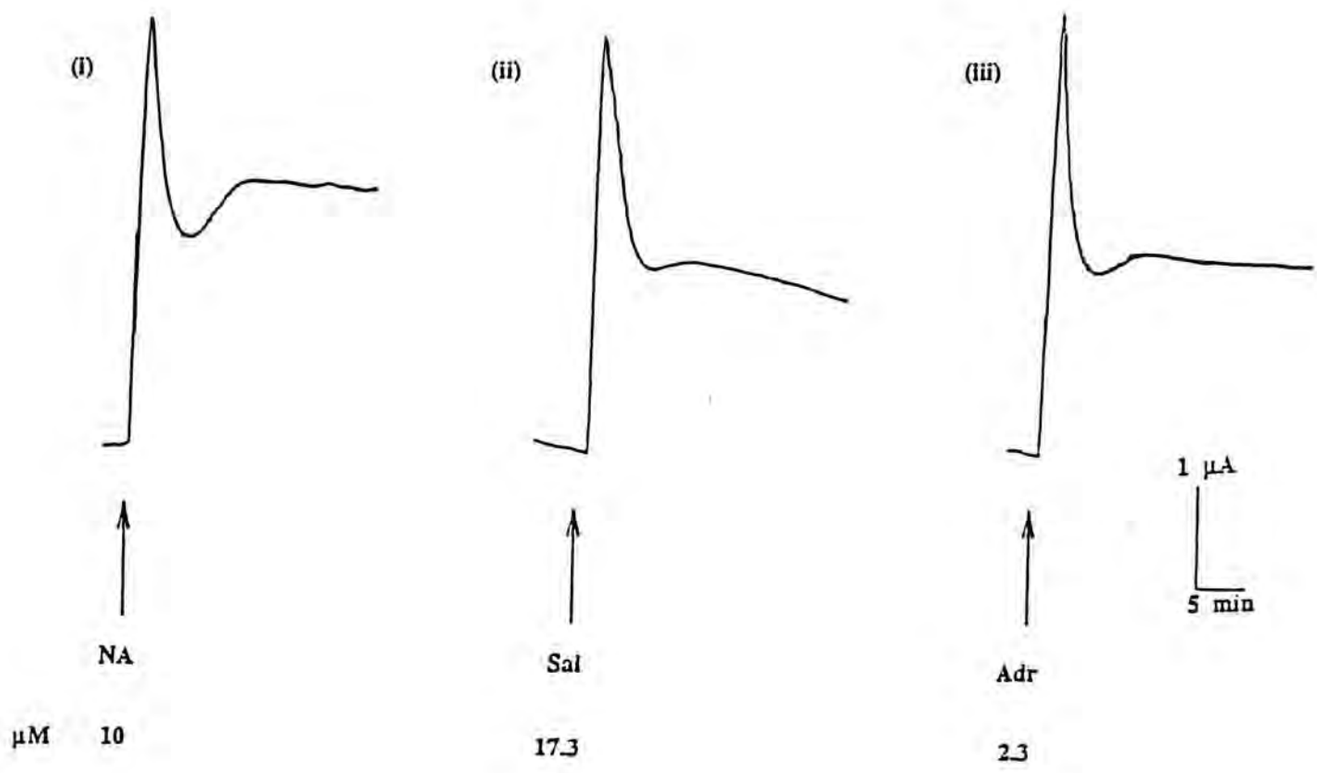


Fig.III.1.1.

Short-circuit current measurement in three separate monolayers (area 0.4 - 0.6 cm^2). The tissues were stimulated with (i) noradrenaline (NA, 10 µM), (ii) salbutamol (Sal, 17.3 µM) and (iii) adrenaline (Adr, 2.3 µM). The agonists were added to the basolateral side of the tissues. The arrows indicate the time at which the drugs were added. Each record is representative of at least six separate experiments.

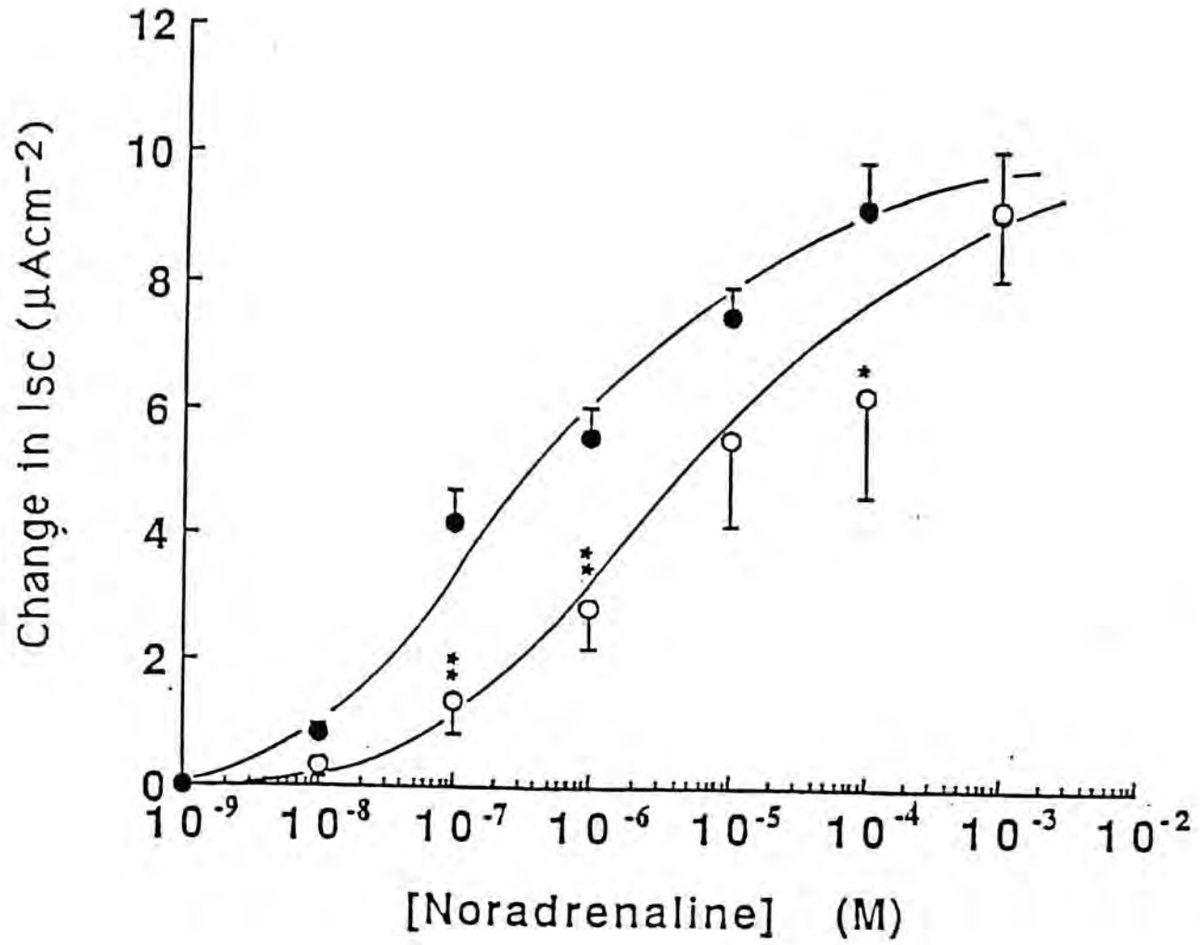


Fig.III.1.2.

Effects of varying the concentration of noradrenaline on the short-circuit current (I_{SC}) response in the absence (•) and presence (o) of 1 μM atenolol. Each data point is the mean of 4 to 6 separate experiments and each error bar represents one S.E.M. Asterisks represent the level of significance when the I_{SC} response to a particular dose of noradrenaline in the presence of atenolol was compared with that in its absence (*, $P < 0.05$; **, $P < 0.01$).

atenolol (1 μM) shifted the dose-response curve to the right with an EC_{50} of 4000 nM. The dose ratio (DR) was thus 13.3 and the pK_B of atenolol was calculated to be 7.1 (See Methods). Fig.III.1.3 shows the dose-response relationships of salbutamol in the absence and presence of a $ß_2$-selective antagonist, butoxamine. The dose-response curve of salbutamol was sigmoidal and the threshold and EC_{50} were 1.7 and 115 nM respectively. Maximum effect was attained at 17.3 μM. Pretreatment with butoxamine (10 μM) shifted the dose-response curve to the right with an EC_{50} of 1050 nM. The DR was 9.1 and the pK_B of butoxamine was calculated as 5.9.

Fig.III.1.4. shows the dose-response relationships of adrenaline in the absence and presence of atenolol or butoxamine. The threshold and EC_{50} of adrenaline were 0.23 and 10 nM respectively and the maximum response was reached at 2.3 μM. Pretreatment with atenolol (1 μM) or butoxamine (10 μM) shifted the dose-response curves to the right with EC_{50}s of 30 and 115 nM respectively. The DR for atenolol was 3 and that for butoxamine was 11.5. The pK_Bs of atenolol and butoxamine were 6.3 and 6.0 respectively.

During the rapidly rising phase of the I_{SC} response to noradrenaline, a brief and fast retraction followed by a rebound increase in I_{SC} was observed (see Fig.III.1.1.i). Fig.III.1.5.i shows the effect of noradrenaline (a non-specific α-agonist in addition to its $ß_1$-selective action) on I_{SC} at expanded time-scale. The retraction and rebound phenomenon manifested as a fast initial spike followed by a delayed and relatively prolonged rise in I_{SC}. The latter corresponded to the peak observed at low chart-speed (see Fig. III.1.1.i). The initial spike lasted for 8-10 seconds and was succeeded by the second component before I_{SC} had returned to the basal level. Similar experiments were performed on other adrenergic agonists. ii & iii show respectively the effects of phenylephrine (an α_1-selective agonist) and adrenaline (a non-specific α-agonist in addition to its ß-action) on I_{SC} at expanded time-scale. Initial spikes were observed with

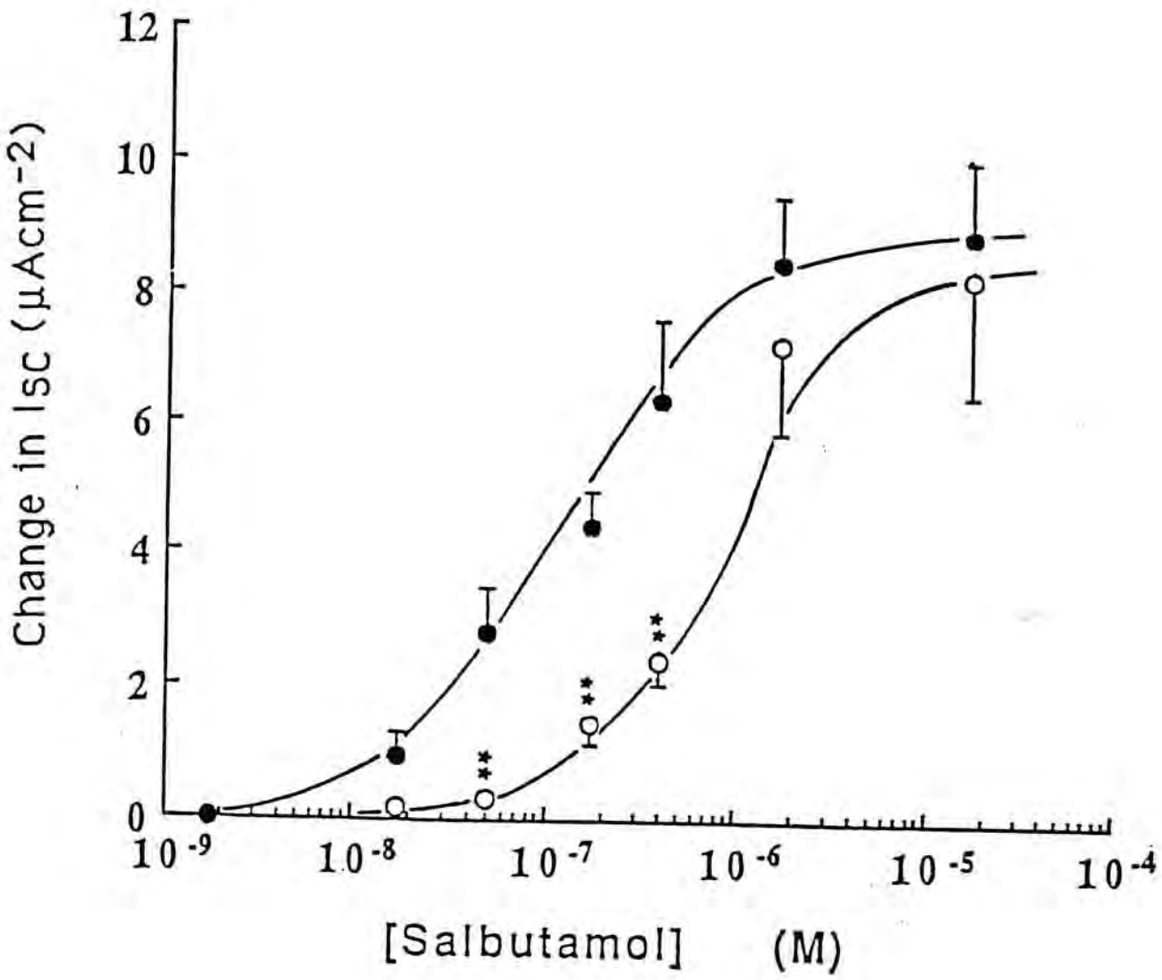


Fig.III.1.3.

Effects of varying the concentration of salbutamol on the short-circuit current (I_{SC}) response in the absence (•) and presence (o) of 10 μM butoxamine. Each data point is the mean of 4 to 6 separate experiments and each error bar represents one S.E.M. Asterisks represent the level of significance when the I_{SC} response to a particular dose of salbutamol in the presence of butoxamine was compared with that in its absence (**, $P < 0.01$).

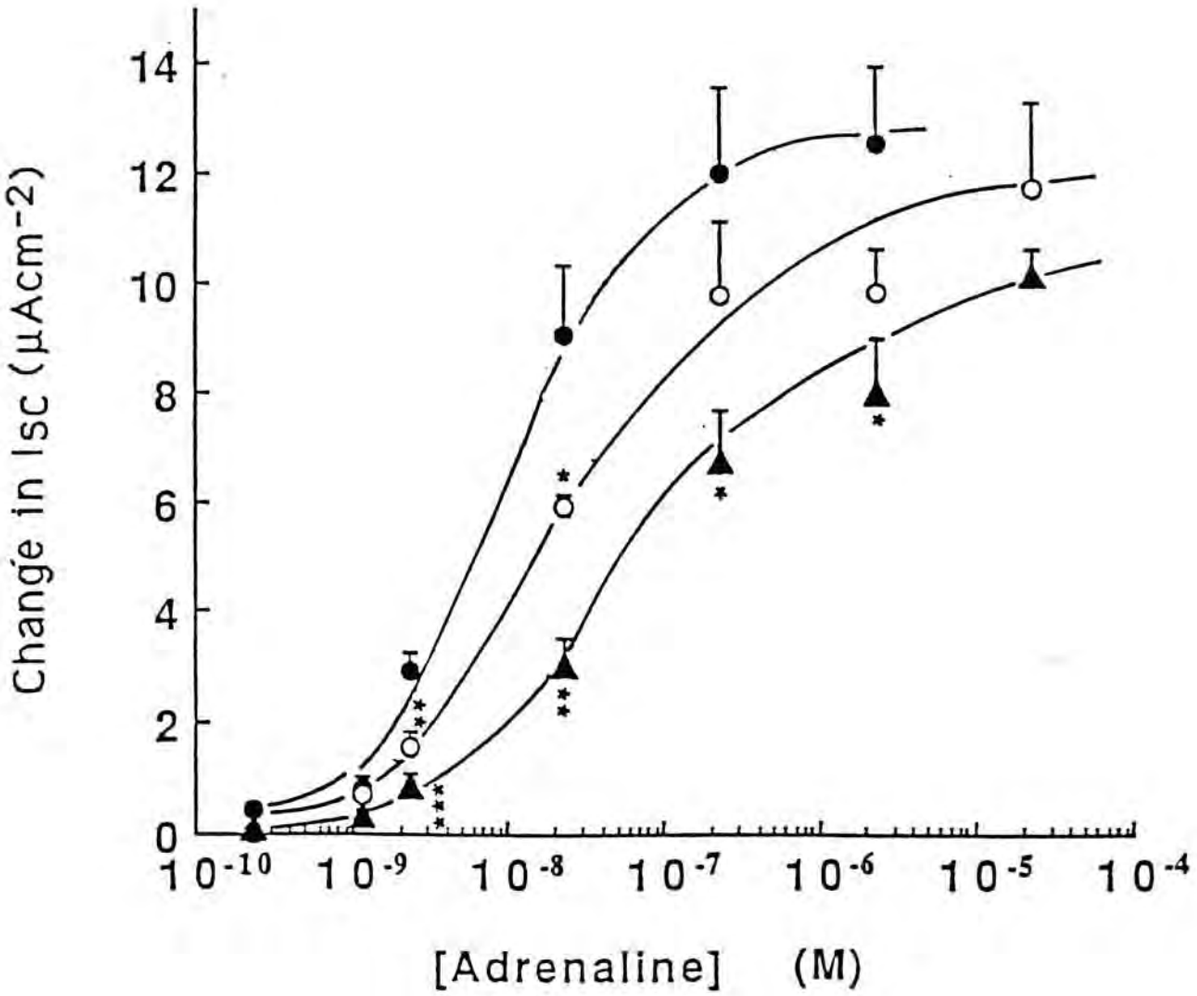


Fig.III.1.4.

Effects of varying the concentration of adrenaline on the short-circuit current (I_{SC}) response in the absence (•) and presence of 1 μM atenolol (o) or 10 μM butoxamine (Δ). Each data point is the mean of 4 to 6 separate experiments and each error bar represents one S.E.M. Asterisks represent the level of significance when the I_{SC} response to a particular dose of adrenaline in the presence of atenolol or butoxamine was compared with that in their absence (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

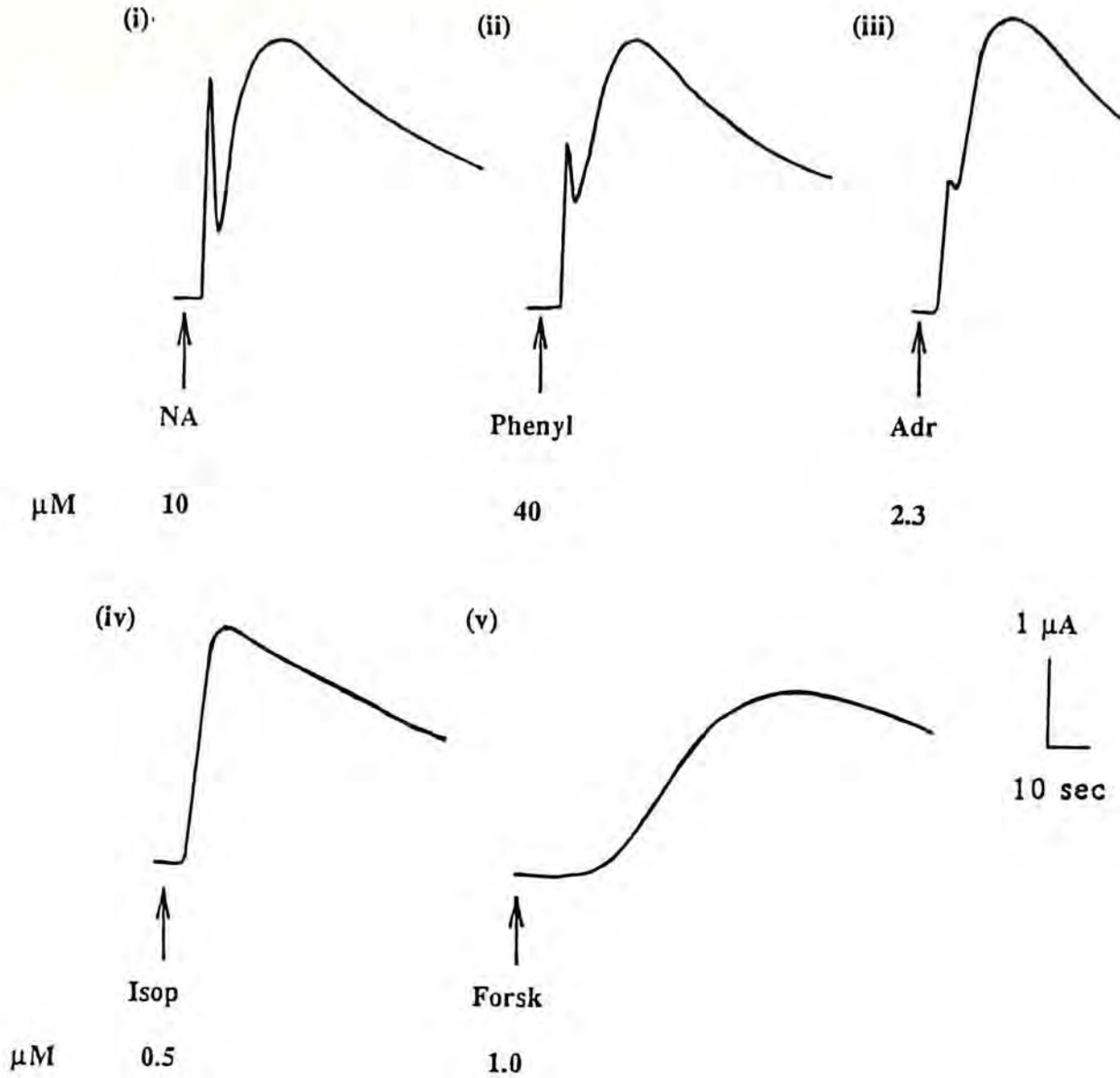


Fig.III.1.5.

Short-circuit current measurement in five separate monolayers (area 0.4 - 0.6 cm^2) at expanded time-scale. The tissues were stimulated with (i) noradrenaline (NA, 10 μM), (ii) Phenylephrine (Phenyl, 40 μM), (iii) adrenaline (Adr, 2.3 μM) (iv) isoprenaline (Isop, 0.5 μM) and (v) forskolin (Forsk, 1.0 μM). The agonists were added to the basolateral side of the tissues. The arrows indicate the time when the drugs were added. Each record is representative of at least six separate experiments.

both agonists. iv & v show respectively the effects of isoprenaline and forskolin on I_{SC}. Isoprenaline is a pure ß-agonist and forskolin is an activator of adenylate cyclase, which forms an integral part of the signal transduction pathway triggered by ß-adrenergic stimulation. The initial spike was not observed upon stimulation with either of them.

Fig.III.1.6 shows the effects of noradrenaline (10 μM) on I_{SC} in the absence (i) and the presence of 0.1 μM (ii) and 0.2 μM (iii) phentolamine, a non-specific α-antagonist. It can be seen that phentolamine at 0.1 μM reduced and at 0.2 μM completely abolished the initial spike triggered by noradrenaline.

Fig.III.1.7. shows the effects of noradrenaline (10 μM) on I_{SC} in the absence (i) or presence of 1 μM (ii) or 10 μM (iii) nadolol, a non-selective ß-antagonist. Nadolol inhibited the second delayed phase of I_{SC} response to noradrenaline in a dose-dependent manner. The initial spike was not affected.

Fig.III.1.8 shows the dose-response relationships of noradrenaline measured as the maximum I_{SC} reached by the initial spike and the second phase of response. Both curves exhibited a sigmoidal profile. The EC_{50} for the initial spike was 2000 nM while that for the second component was 300 nM. The threshold doses required to elicit the initial spike and the second component were 10 and 1 nM respectively.

Stimulation of α_1-adrenoceptors is associated with Ca^{2+} release from intracellular store (Exton, 1985). Studies were carried out to investigate the effects of perturbing intracellular Ca^{2+} homeostasis on the initial spike triggered by noradrenaline. Fig.III.1.9 shows that loading the tissue with BAPTA/AM, a cell permeant Ca^{2+} chelator, reduced the magnitude of the initial spike in a dose-dependent fashion. The seond phase of response was not affected. Microfluorimetric studies were performed to measure the $[Ca^{2+}]_i$

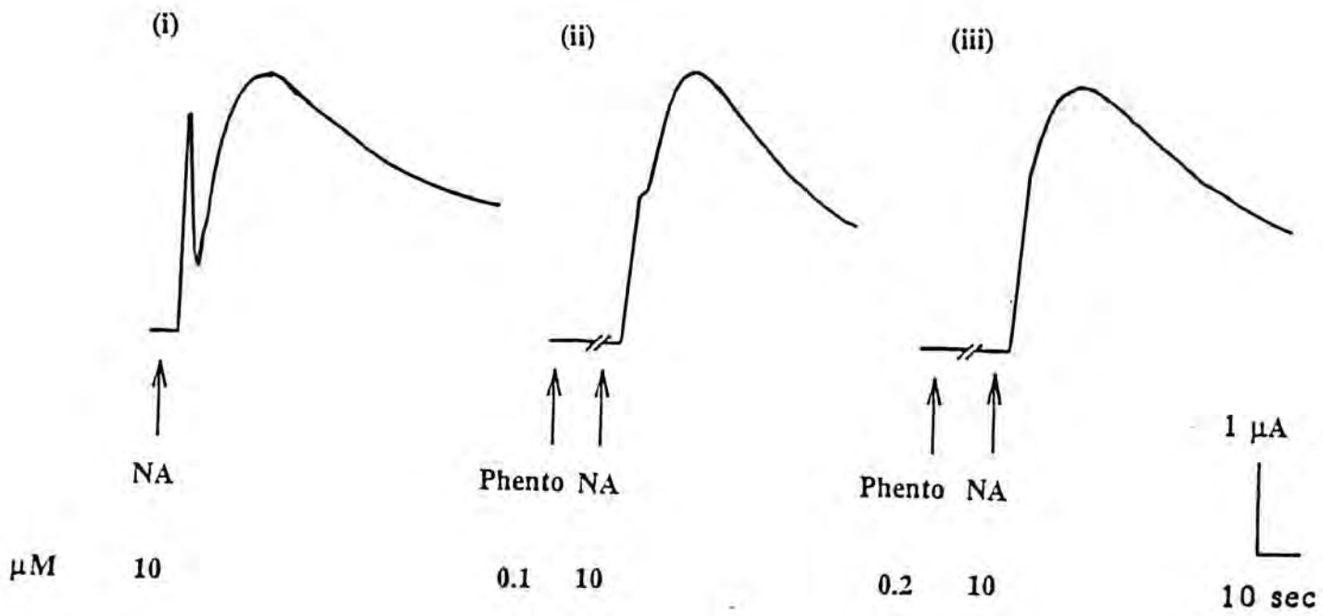


Fig.III.1.6.

Short-circuit current measurement in three separate monolayers (area 0.4 - 0.6 cm^2) at expanded time-scale. The tissues were stimulated with noradrenaline (NA, 10 µM) in the (i) absence and presence of (ii) 0.1 µM or (iii) 0.2 µM phentolamine. The agonists were added to the basolateral side of the tissues. The arrows indicate the time when the drugs were added. Each record is representative of at least 4 separate experiments.

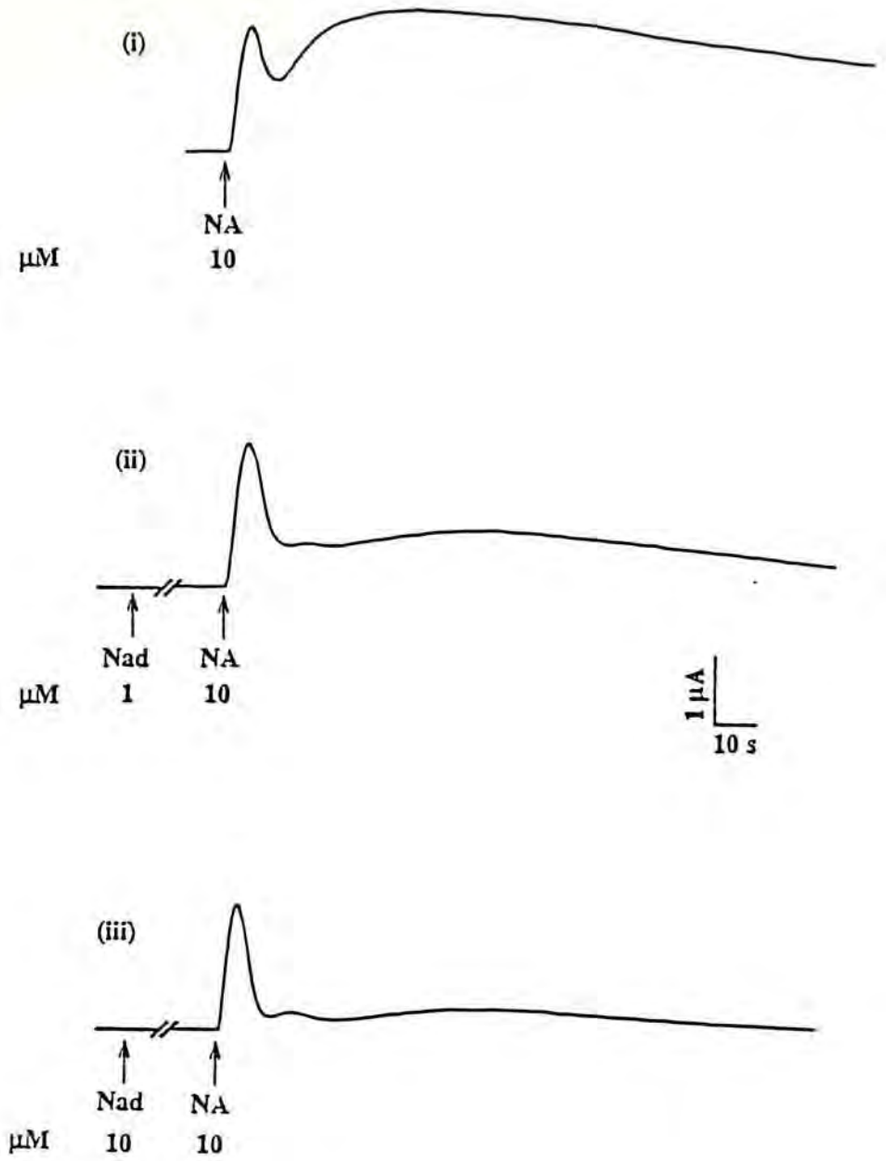


Fig.III.1.7.

Short-circuit current measurement in three separate monolayers (area 0.4 - 0.6 cm^2) at expanded time-scale. The tissues were stimulated with noradrenaline (NA, 10 μM) in the absence (i) or presence of 1 μM (ii) or 10 μM (iii) nadolol (Nad). The arrows indicate the time when the drugs were added. Each record is representative of 3 separate experiments.

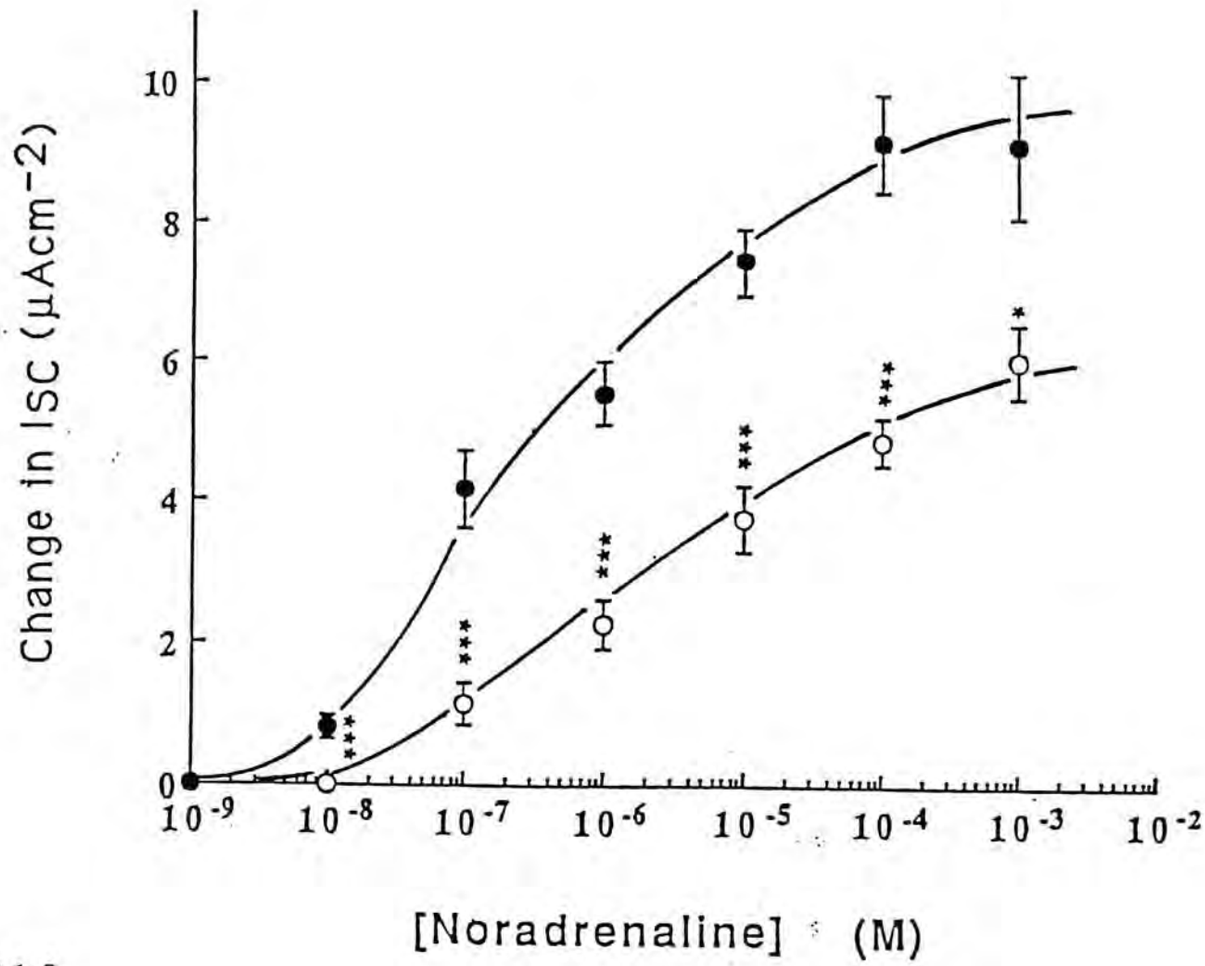


Fig.III.1.8.

Effects of varying the concentration of noradrenaline on the short-circuit current (I_{SC}) response expressed as the maximum I_{SC} reached by the initial spike (o) and the second peak (•). Each data point is the mean of 4 to 6 separate experiments and each error bar represents one S.E.M. Asterisks represent the level of significance when the maximum SCC reached by the initial spike at a particular dose of noradrenaline was compared with that reached by the second peak (*, $P < 0.05$; ***, $P < 0.001$).

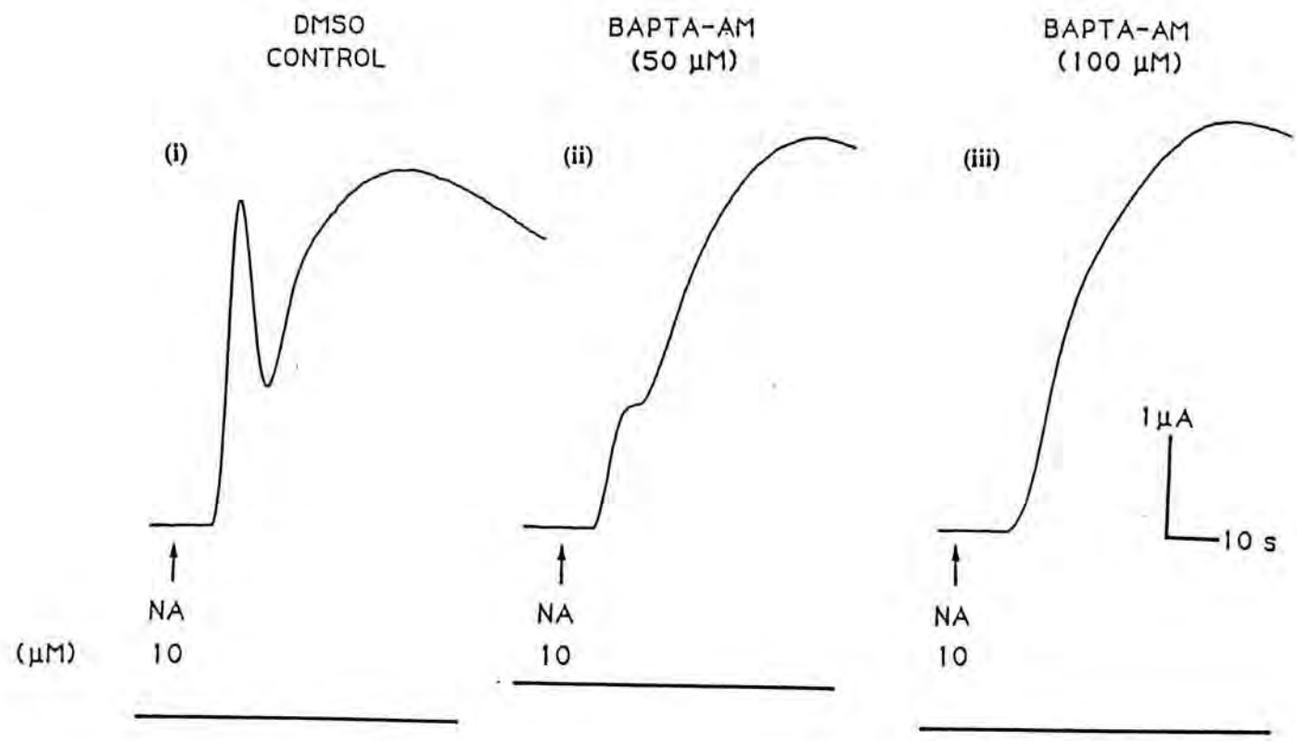


Fig.III.1.9

Effects of a cell-permeant Ca^{2+} chelator BAPTA/AM on the I_{SC} response to noradrenaline (NA, 10 μM) at expanded time scale. (i) The monolayer was incubated with DMSO before noradrenaline stimulation as a control. (ii) The monolayer was incubated with 50 μM and (iii) 100 μM BAPTA/AM before noradrenaline stimulation. The arrows indicated the time when noradrenaline was added. Each record is representative of 5 separate experiments.

changes triggered by noradrenaline. Fig.III.1.10. shows the effects of sequential stimulations with noradrenaline and thapsigargin, Tg, on $[Ca^{2+}]_i$ in single epididymal cells. Thapsigargin has been shown to inhibit the microsomal Ca^{2+}ATPase, leading to depletion of internal Ca^{2+} store (Thastrup et al., 1990). The cells were bathed in HEPES-buffered solution containing 2.5 mM Ca^{2+} plus 1 mM La^{3+} (appendix III). Noradrenaline gave rise to a Ca^{2+} spike and subsequent Tg stimulation led to an additional transient rise in $[Ca^{2+}]_i$. When the sequence of stimulation was reversed, prior stimulation with Tg led to a transient rise in $[Ca^{2+}]_i$ but abolished the $[Ca^{2+}]_i$ response to subsequent noradrenaline stimulation. This suggested that the Ca^{2+} pool releasable by noradrenaline was functionally smaller than but overlapped with that by Tg.

Fig.III.1.11 shows the effects of noradrenaline on $[cAMP]_i$. The basal $[cAMP]_i$ was 34.4 ± 2.07 pmol/well. 5 minutes after noradrenaline stimulation, $[cAMP]_i$ increased to 116.0 ± 8.78 pmol/well ($P < 0.001$, unpaired t-test). The $[cAMP]_i$ response to noradrenaline could be abolished by pretreatment with nadolol (50 μM), suggesting that it was mediated by ß-adrenergic stimulation.

Plate 2 shows the immunfluorescence staining for dopamine ß-hydroxylase, suggesting the presence of adrenergic fibres in the rat cauda epididymis. (a) Adrenergic fibres were found in the tubular smooth muscle (Sm), but not in the lumen (L) of the epididymal tubules. Some fibres were found to penetrate into the epithelium (arrows) and ramify over the basal aspect of the epithelial cells (arrows). (b) Adrenergic fibres were seen as a plexus around an arteriole in the peritubular spaces.

Discussion

It has been reported in the epididymal (Wong & Chan, 1988) and tracheal epithelium (Al-Bazzaz & Cheng, 1979) that Cl^- secretion could be stimulated by ß-

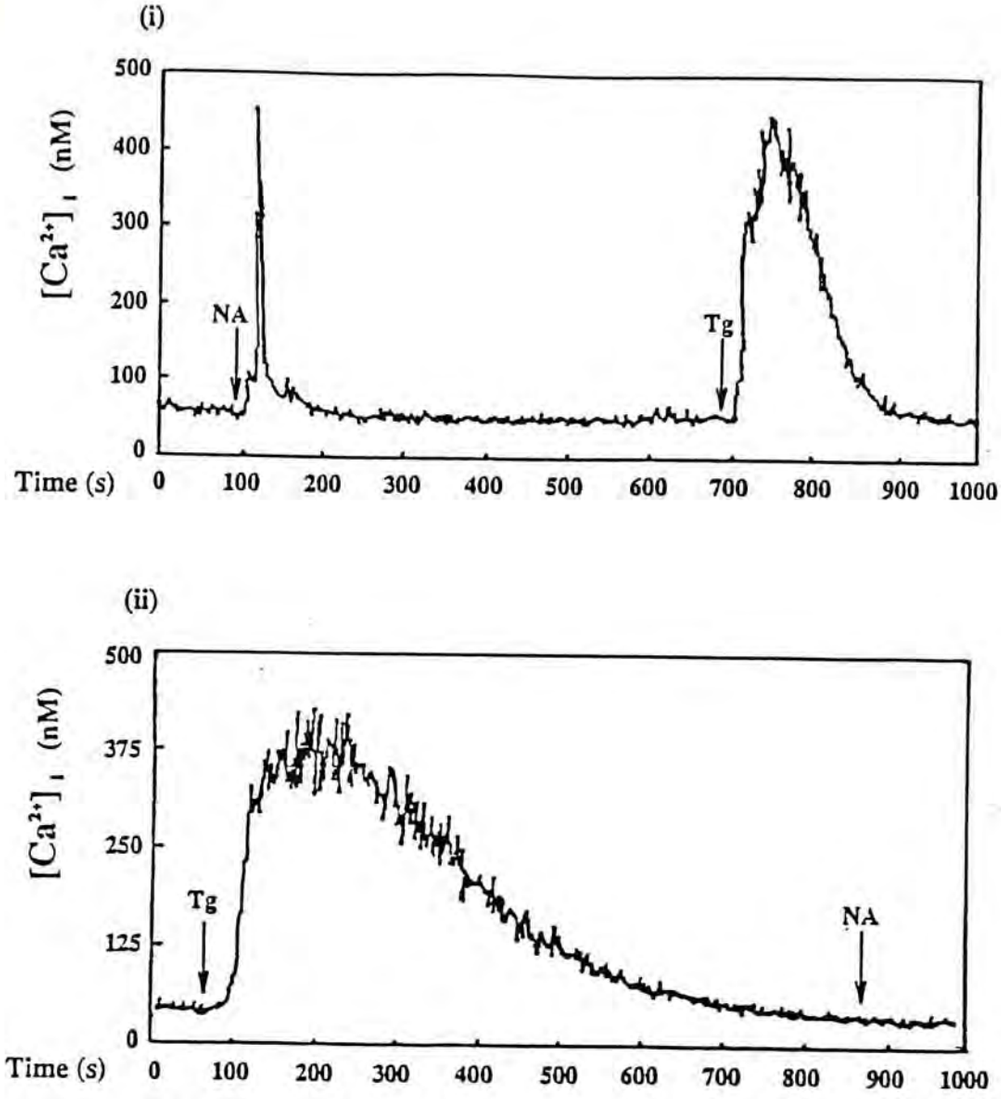


Fig.III.1.10

Effects of sequential stimulations with noradrenaline and thapsigargin on $[Ca^{2+}]_i$ in single epididymal cells in HEPES-buffered solution containing 2.5 mM free Ca^{2+} plus 1 mM La^{3+}. (i) The cell was stimulated with noradrenaline (NA, 10 μM) followed by thapsigargin (Tg, 1 μM). (ii) The cell was stimulated with thapsigargin (Tg, 1 μM) followed by noradrenaline (NA, 10 μM). Each record is representative of at least 5 experiments.

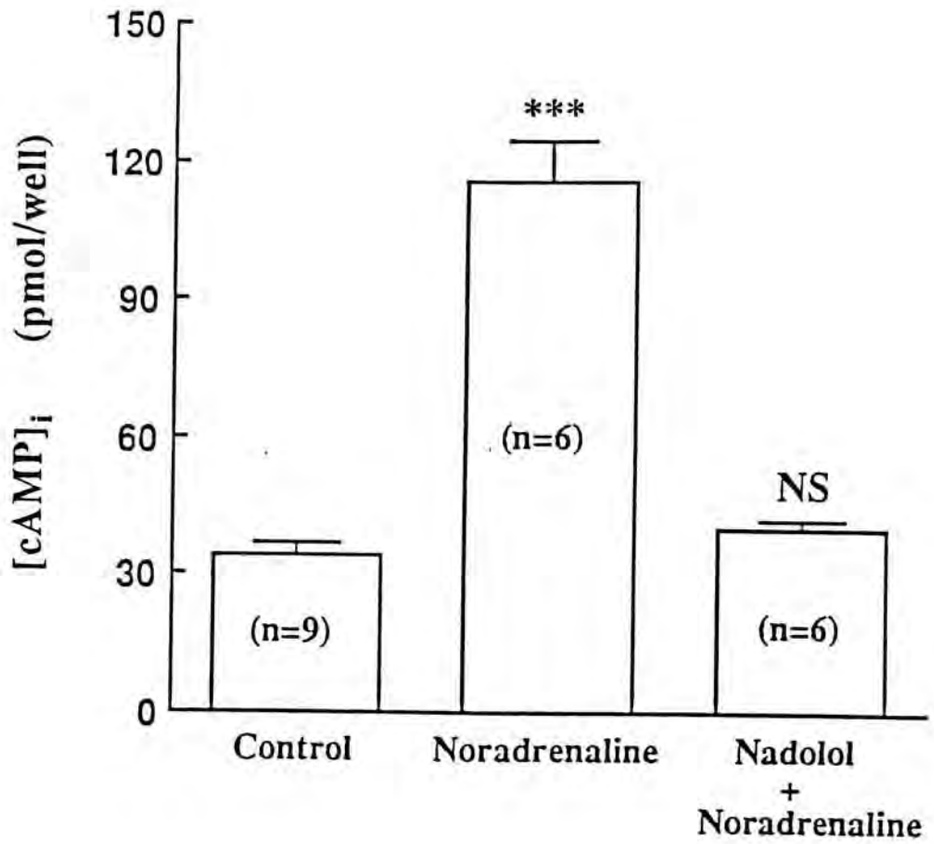


Fig.III.1.11

Effects of noradrenaline (10 μM) on intracellular cAMP concentration ($[cAMP]_i$). Noradrenaline elicited a rise in $[cAMP]_i$ which was abolished by prior treatment with an non-selective ß-antagonist, nadolol (50 μM). Each error bar represents one S.E.M.. (*** $P < 0.001$, NS no significance, Student's unpaired t-test).

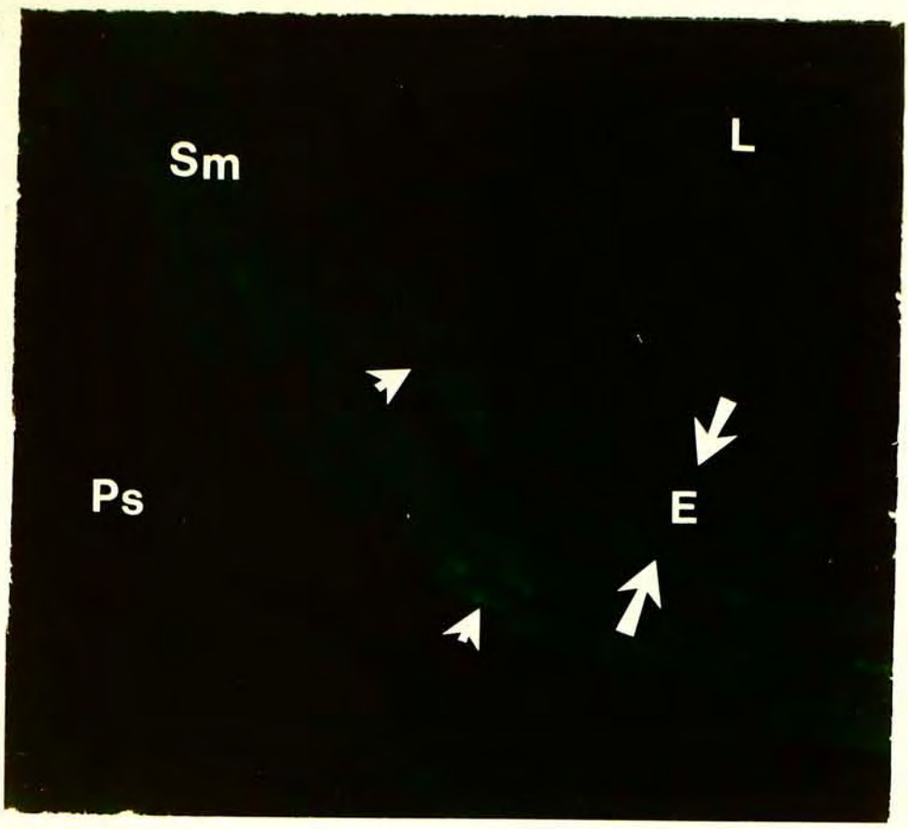
Plate 2

Immunofluorescence staining of noradrenergic fibres in the cauda epididymidis of rats. The enzyme involved in the synthesis of noradrenaline from dopamine, namely, dopamine ß hydroxylase (DBH) was used as a marker for the fibres (rabbit anti-rat DBH, 1:50 dilution).

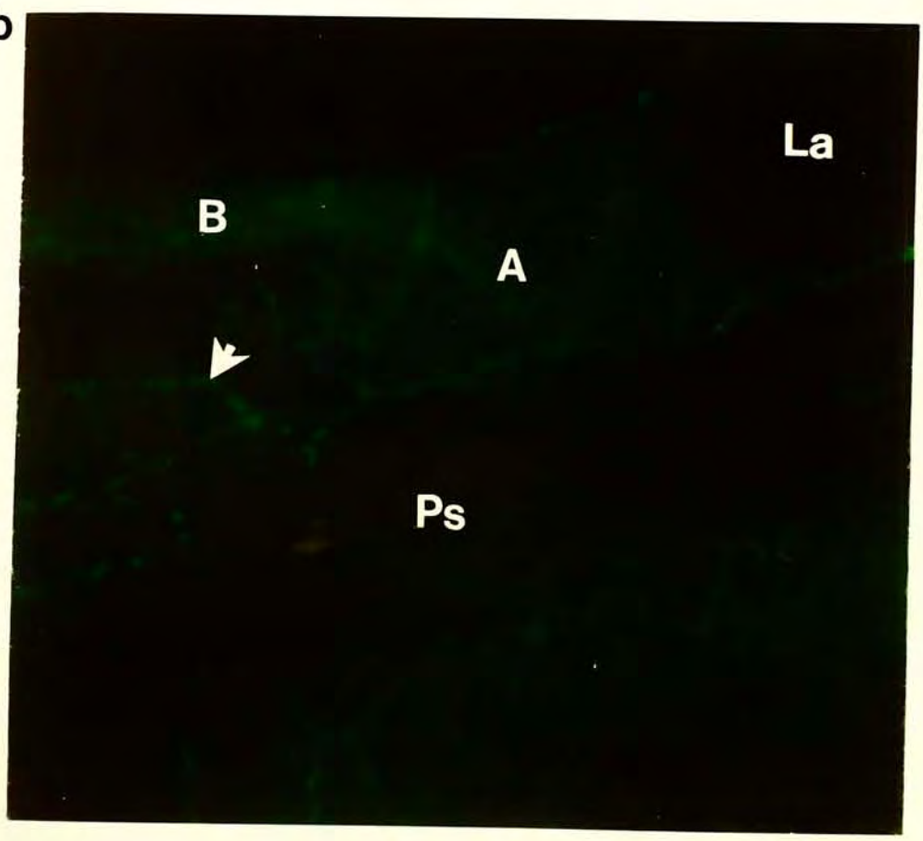
(a) Nerve fibres (arrow heads) were seen in the basal aspect of the epithelium (E) and peritubular smooth muscle layer (Sm). No immunostaining was found in the lumen of the tubule (L) Ps: Peritubular space (x 400).

(b) Nerve fibres were also found in peritubular arteriole (A). No immunostaining was found in the lumen of the arteriole (La) (x400).

a



b



adrenergic stimulation. The present study showed that both $ß_1$- and $ß_2$- receptors were involved. To investigate the receptor subtypes, noradrenaline and salbutamol were used as selective $ß_1$- and $ß_2$-adrenergic agonists respectively (Nahorski, 1981). Although a detailed Schild Plot analysis has not been performed, the pK_B of 7.1 and 5.9 determined from a single concentration of atenolol (1 μM) and butoxamine (10 μM) respectively were comparable to those reported in studies using atrial tissues (Leclerc et al., 1981; O'Donnell & Wanstall, 1983). These suggested that the effects of atenolol and butoxamine were due to specific inhibition of $ß_1$- and $ß_2$-adrenoceptors respectively. The I_{SC} response to adrenaline could also be inhibited by atenolol or butoxamine, suggesting that it was mediated by both $ß_1$- and $ß_2$- adrenoceptor stimulation.

ß-adrenergic stimulation is associated with a rise in intracellular cAMP concentration (Levitzki, 1988). The present study showed that noradrenaline elicited a rise in $[cAMP]_i$, which could be abolished by pretreatment with a non-selective ß-antagonist, nadolol (Fig.III.1.11). The latter was also found to inhibit the second component of the I_{SC} response to noradrenaline at expanded time-scale (Fig. III.1.7.), supporting the notion that increase in $[cAMP]_i$ upon ß-adrenergic stimulation leads to activation of Cl^- secretion across the epithelium.

The present study also provided evidence for the existence of α_1-receptors in the epididymal epithelium. The α_1-selective agonist phenylephrine, as well as the non-selective a-agonists noradrenaline and adrenaline, gave rise to an initial spike which could be blocked by phentolamine, a non-selective α-antagonist. It is interesting that in addition to the α_1-mediated initial spike, the I_{SC} response to phenylephrine exhibited a prominent second component suggesting that in the epididymis, phenylephrine possessed significant ß-adrenergic effect in addition to its specific α_1-action. The ß-effects of phenylephrine on I_{SC} measurement have also been reported in tracheal epithelium (Bainbridge et al., 1989).

It was also found that the intial spike triggered by noradrenaline could be reduced by a cell-permeant Ca^{2+} chelator, BAPTA/AM in a dose-dependent fashion. BAPTA/AM diffused into the cells and was hydrolysed by cytosolic esterase to release free BAPTA. The latter chelated intracellular free Ca^{2+}, thereby reducing the secretory response which was mediated by increase in $[Ca^{2+}]_i$. The results were not unexpected as α_1-adrenergic agonists are prototypes of Ca^{2+}-mobilizing agonists (Exton, 1985). The existence of α_1-receptors is further supported by the fact that noradrenaline triggered a transient rise in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ response to noradrenaline was attributed to Ca^{2+} release from intracellular store because the influx and efflux of Ca^{2+} across the plasma membrane have been inhibited by La^{3+}, which formed a 'Ca^{2+} seal' over the plasma membrane. Noradrenaline might act on α_1-adrenoceptors and the ligand-receptor complex initiated the hydrolysis of PIP_2 into IP_3 and DG via the coupling G protein (See chapter I.3). IP_3 bound onto internal Ca^{2+} store, causing release of Ca^{2+} into the cytosol. This was supported by the observation that the $[Ca^{2+}]_i$ response to noradrenaline could be abolished by prior stimulation with Tg (Fig.III.1.10), which has been found to deplete the IP_3-sensitive Ca^{2+} store in parotid acinar cells (Takemura et al., 1989).

The demonstration of α_1-, β_1- and β_2-adrenergic receptors suggested that Cl^- secretion by the rat cauda epididymidis was under both neural and humoral controls. Noradrenaline released from nerve terminals might act on α_1- and β_1-adrenoceptors on the epithelium to stimulate Cl^- secretion (Wong et al., 1992). Immunoreactive dopamine ß-hydroxylase could be detected in the epithelium and this might indicate the presence of adrenergic fibres ramifying over the basal aspect of the epithelial cells (Plate 2), supporting the notion that sympathetic innervation served a secretomotor function. On the other hand, Cl^- secretion was also stimulated by adrenaline and the threshold dose required to elicit a response was close to the adrenaline concentration in the systemic circulation (Eisenhofer et

al., 1985). Circulating adrenaline activated various adrenergic receptors, including β_2-receptors (Davis et al., 1990) on the epithelium to stimulate Cl^- secretion.

The neurohumoral control of Cl^- secretion plays a role in the maintenance of a unique and specific microenvironment in the epididymis on which the maturation and storage of spermatozoa depend (Jenkins et al., 1980). Disruption of the control mechanism by, for instance, sectioning the sympathetic nerve supply to the epididymis has been shown to adversely affect sperm transport and motility (Billups et al., 1990a). Sympathetic denervation might lead to functional tubular obstruction (Billups et al., 1990b), a reflection of defective fluid transport in the epididymis (Wong, 1990).

The present study demonstrated the co-existence of α_1-, β_1- and β_2-adrenoceptors in a single exocrine tissue. Co-expression of α_1- and β_2-adrenoceptors has also been reported in the MDCK cells (Slivka & Insel, 1987). It is interesting that the α_1- and β-mediated Cl^- secretion in the epididymis follows different temporal profiles. The α_1-response has a faster onset and more transient action while the β-response has a relatively delayed onset but more prolonged effects. Similar temporal sequence of α_1-and β-mediated cellular responses has been found in MDCK cells (Breuer et al., 1988). The present study did not establish whether both responses are mediated by a single epithelial cell, as in MDCK cells (Breuer et al., 1988) or by two separate epithelial cell types with different signal transduction mechanisms.

Reagents	Procedures	Time
10 % formalin	Fixation	8 min
0.1 M PBS	Washing	10 min x 3
1:50 rabbit anti-DBH antiserum	Staining	Overnight 4°C
0.1 M PBS	Washing	10 min x 3
1: 100 FITC-conjugated goat anti-rabbit IgG	Staining	60 min Room Temp.
0.1 M PBS	Washing	10 min x 3
Glycerol	Mounting	—

Table III.1.1

Procedures for immunofluorescence staining

Chapter III.2.

Local control of electrogenic chloride secretion in rat epididymis --- The role of the calcitonin gene-related peptide

Summary

The present study investigated local control of Cl^- secretion by rat epididymal epithelium. The calcitonin gene-related peptide (CGRP) was chosen as an example because it has been shown to affect electrolyte and fluid secretion in various exocrine tissues (Cox et al, 1989; Ekström et al., 1988; Mathison & Davison, 1989). CGRP gave rise to a dose-dependent increase in I_{SC} and the effect was restricted to the basolateral aspect of the cells.The I_{SC} response was inhibited in a competitive fashion by a receptor antagonist CGRP(8-37). Receptors for CGRP were rapidly desensitized upon repeated stimulations. Intracellular cAMP showed a significant time- and dose-dependent increase upon CGRP stimulation Immunofluorescence staining of CGRP was found in the epithelium of the rat cauda epididymidis. It is suggested that Cl^- secretion in the epididymis may be regulated by local factors (i.e. CGRP) which are synthesized by the epithelial cells and are released in a paracrine fashion to exert its effects on electrolyte and fluid secretion thereby providing an optimal microenvironment for the maturation and storage of spermatozoa.

Introduction

In addition to neurohumoral control (Chapter III.1.), Cl^- secretion in the epididymis may be regulated by local factors which are synthesized by the epithelium and are released in a paracrine fashion to exert effects on neighbouring epithelial cells. This provides long-term regulation of the epithelium to ensure the maintenance of a stable microenvironment for the maturation and storage of spermatozoa. The identities of local factors are unknown but they may be numerous to enable fine-tuning of the epithelial functions. The calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide produced by the alternative processing of the primary transcript of the calcitonin gene (Amara et al., 1985). The CGRP was suspected as one of these local factors because it has been found to be widely distributed in the body (Rosenfield et al., 1983) and it affected fluid and electrolyte transport in various exocrine tissues, for instance, the salivary gland (Ekström et al., 1988), the stomach (Helton et al., 1989), the pancreas (Mulholland et al., 1989), the duodenum (Lenz and Brown, 1990) as well as the colon (Cox et al., 1989). The present study investigated the role of the CGRP in Cl^- secretion by rat epididymis.

Methods

Techniques of tissue culture, short-circuit current measurement, intracellular cAMP measurement as well as immunofluorescence staining have been described (see Chapter II.1-4. & III.1.). When the time-dependence of the $[cAMP]_i$ response to the CGRP was studied, the epididymal cells were exposed to the peptide for 0-10 minutes. When the dose-dependence was studied, the cells were allowed to expose to various concentration of the peptide for 6 minutes. The primary antibody used for immunofluorescence was rabbit anti-CGRP antiserum (1:100 dilution). The secondary antibody was FITC-conjugated goat-anti rabbit antiserum (1:200 dilution). The staining procedures were otherwise identical to those described in Chapter III.1. For short-circuit current measurement, the monolayers were bathed in normal K-H solution (Appendix II).

Results

Fig.III.2.1 shows the effects of CGRP on I_{SC} when it was added to the apical and the basolateral side of the epithelium. Addition of 8.5 nM CGRP to the basolateral side led to a profound increase in I_{SC} (Fig.III.2.1.ii) while on the apical side, the effect of the peptide was negligible at either 3.1,16.5 or 33 nM (Fig.III.2.1.i.). In all subsequent experiments, the peptide was added to the basolateral aspect of the tissue. Fig.III.2.2. shows the typical I_{SC} response to varying concentrations of CGRP and the responses after the monolayer has been treated with 400 nM of a CGRP receptor antagonist $CGRP_{(8-37)}$, which consists of the 8th to 37th amino acid residues of the parent peptide (Chiba et al., 1989). CGRP gave rise to a transient increase in I_{SC} which returned to the basal level after 4-5 minutes. The transient characteristic was more apparent with increasing doses. Pretreatment with the antagonist reduced the effect of CGRP. Fig.III.2.3. shows dose-response curve of CGRP with and without antagonist pretreatment. The I_{SC} response to CGRP was defined as the maximum increase in I_{SC} upon stimulation with the peptide. The EC_{50} pf CGRP was 15 nM and the maximum I_{SC} response was 5.8 μAcm^{-2}. Pretreatment with the $CGRP_{(8-37)}$ shifted the dose-response curve to the right.

To investigate the receptor desensitization phenomenon for CGRP, experiments were carried out to study the effects on I_{SC} upon repetitive stimulations with CGRP. Fig.III.2.4.i. shows that 131 nM CGRP gave rise to an increase in I_{SC} but abolished the effect of the subsequent stimulation with the same concentration of the peptide. However, the I_{SC} response to adrenaline (0.23 μM) was not affected. Fig.III.2.4.ii. shows the control experiment in which the tissue was only stimulated with adrenaline and the magnitude of response was similar to that in (i). Therefore, the receptors for CGRP was rapidly desensitized after prior stimulation with the peptide. Fig.III.2.5. shows the effects of adrenaline and forskolin (Chapter III.1.) pretreatments on the I_{SC} response to CGRP. Both of them greatly increased I_{SC} but nearly abolished the effect of subsequent CGRP

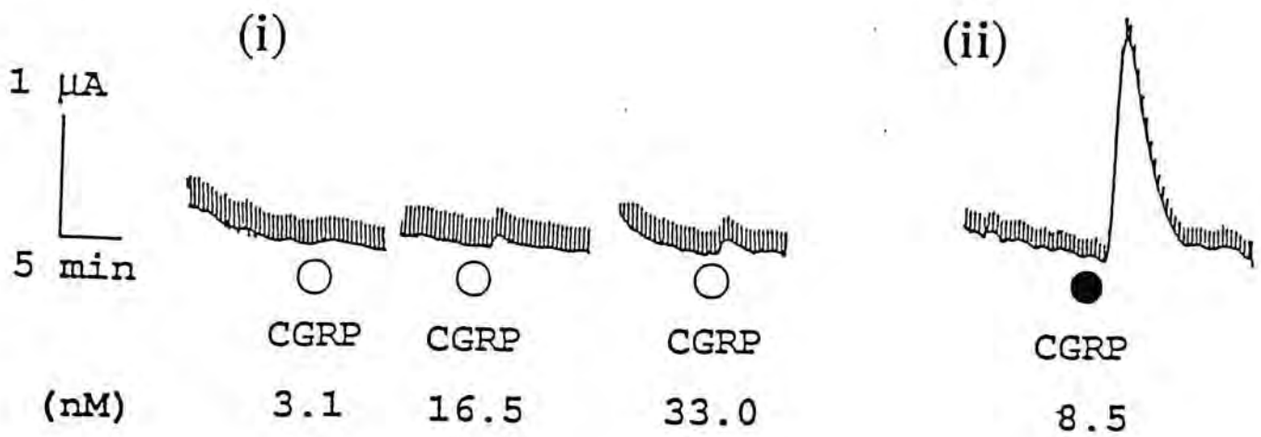


Fig.III.2.1.

Short-circuit current measurement in four separate epididymal monolayers. (i) CGRP added to the apical side of 3 separate monolayers at the concentrations shown. (ii) CGRP (8.5 nM) added to the basolateral side.

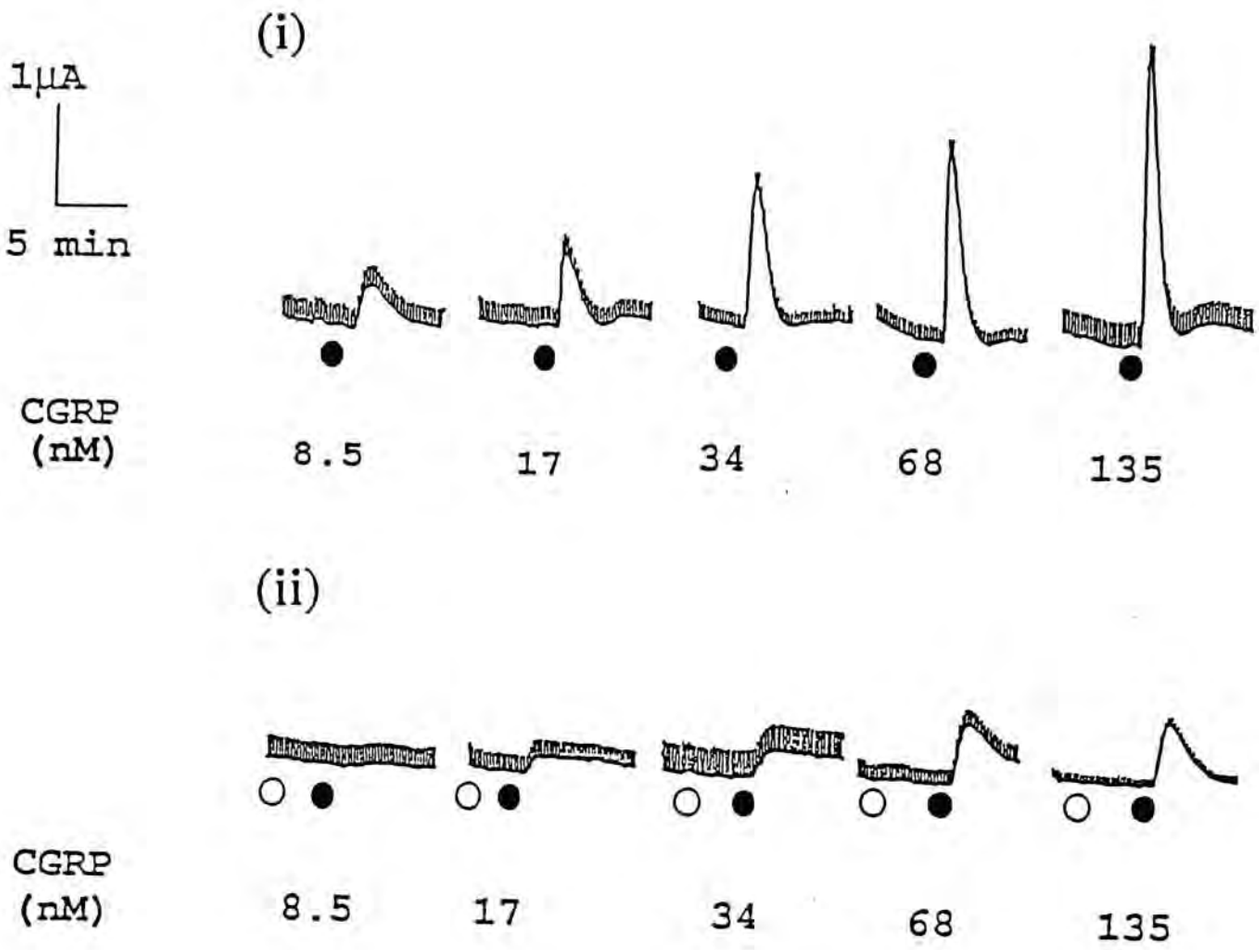


Fig.III.2.2.

Short-circuit current measurement of 10 separate monolayers. In (i) and (ii) CGRP was added to the basolateral side (•) at the concentration shown. In (ii), monolayers were pretreated with a basolateral application of CGRP receptor antagonist, CGRP(8-37) (o) prior to the addition of CGRP.

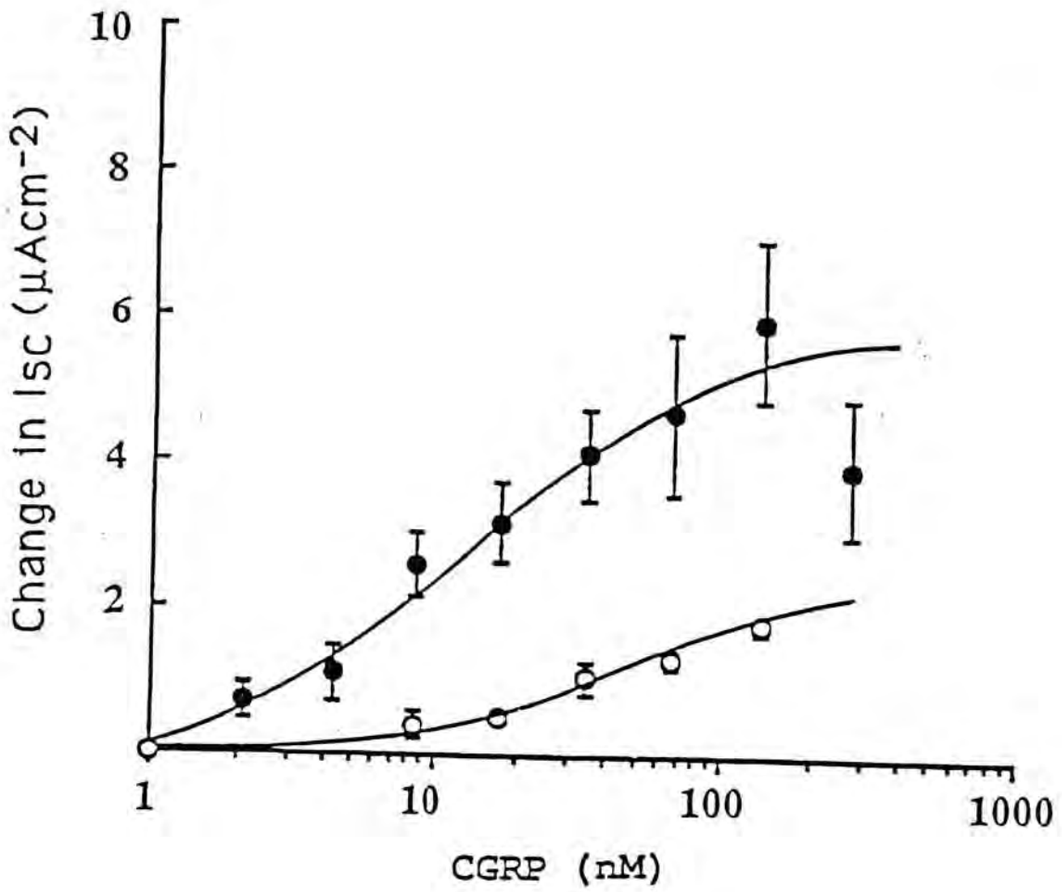


Fig.III.2.3.

Effect of various concentrations of CGRP on changes in short-circuit current. CGRP was applied basolaterally to tissues with (o) or without (•) pretreatment with the CGRP antagonist, CGRP(8-37). Values are means ± s.e.m. for 4 to 6 cultures.

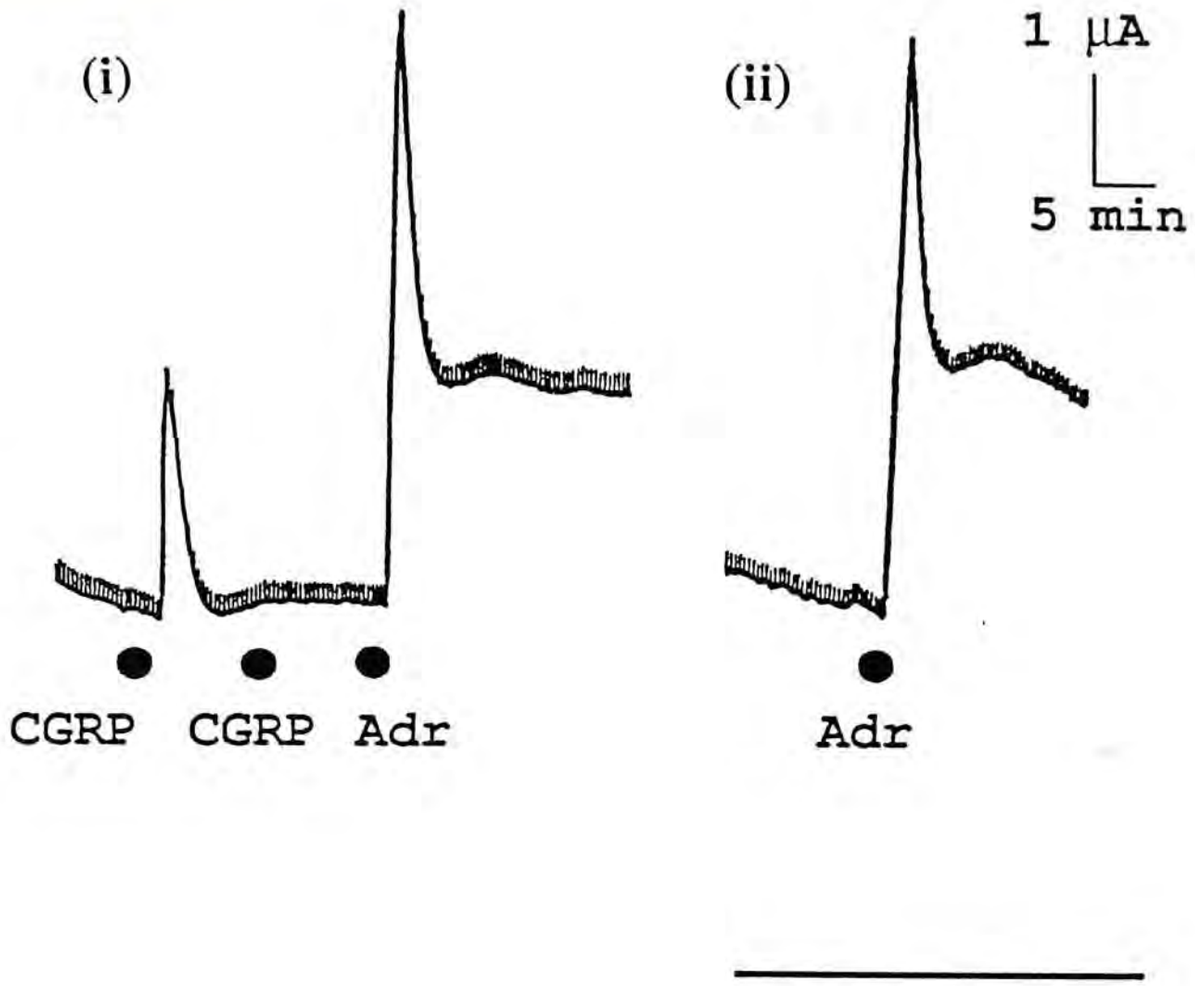


Fig.III.2.4.

Short-circuit current measured simultaneously in two separate monolayers of rat epididymal cells. In (i), monolayer was first stimulated with basolateral application of CGRP (131 nM). This resulted in a rise in short-circuit current. When the current had returned to the basal level, the monolayer was stimulated with a second addition of CGRP (131 nM) added basolaterally. Note the response to the first addition has abolished the response to the second addition. The tissue was subsequently stimulated with adrenaline (Adr, 0.23 μM) added basolaterally. In (ii), monolayer was stimulated with adrenaline (Adr, 0.23 μM) alone. The horizontal lines indicate zero short-circuit current.

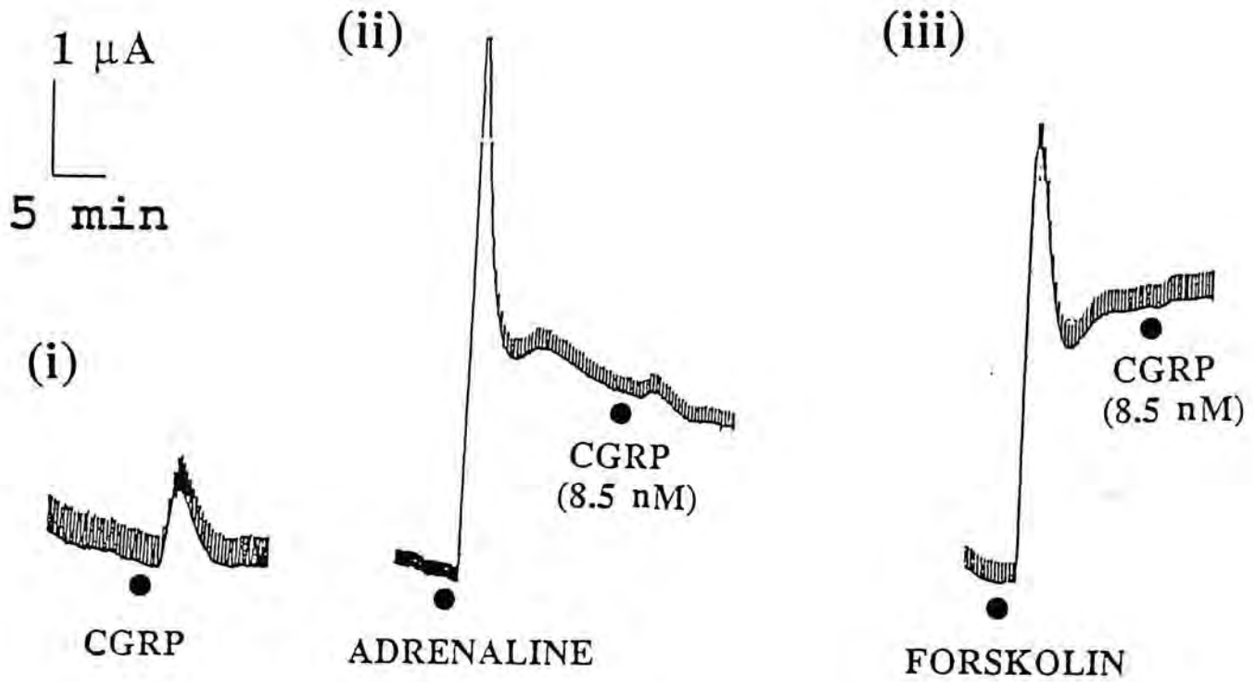


Fig.III.2.5

Short-circuit current measured in three separate monolayers of rat epididymal monolayers. In (i), monolayer was stimulated with CGRP (8.5 nM) alone. In (ii), monolayer was stimulated with adrenaline (Adr, 0.23 μM) followed by CGRP (8.5 nM). In (iii), monolayer was stimulated with forskolin (Fors, 10 μM) followed by CGRP (8.5 nM). All agonists were added to the basolateral side of the tissues.

stimulation. Since adrenaline and forskolin increase intracellular cAMP level (Wong & Huang 1989) and in their presence, the I_{SC} response to CGRP was nearly abolished, the secretory response to CGRP might involve cAMP as a second messenger.

Fig.III.2.6. shows the time course of $[cAMP]_i$ upon stimulation with 500 nM CGRP. Under basal condition (time=0), the average intracellular cAMP content was 38.9 ± 3.4 pmol/well (n = 12 cultures). It started to rise 2 min. after CGRP addition. A plateau level at 68.5 ± 6.5 pmol/well (n = 12 cultures) was reached at 6 min. and thereafter it fell to a value not significantly different from that at time zero. Fig.III.2.7. shows the dose-response relationship between CGRP and $[cAMP]_i$. A maximum rise in $[cAMP]_i$ of 82.2 ± 15 pmol/well was observed at 1512 nM CGRP and the EC_{50} was 150 nM. The figure also shows the dose-response relationship of I_{SC}. It can be seen that the curve of $cAMP_i$ was parrallel to that of I_{SC} but was shifted to the right.

(a), (b) and (c) in plate 3 show respectively the photomicrographs of the initial segment, the caput and corpus epididymidis of rats. No immunostaining for CGRP could be found in the lumen (L), the epithelium of the epididymal tubules (E) or the peritubular space (Ps). In (d), a portion of a tubule in the rat cauda epididymidis is shown. Intense immunofluorescence was observed in the epithelium (E) as defined by the white arrows. No immunoreactivity was found in the lumen of the tubule (L), the smooth muscle (Sm) and the peritubular space (Ps). (e) shows the photomicrograph of a similar section in which the primary antibody was preadsorbed with 40 μM of CGRP before incubation. Noimmunoreactivity was found in the epithelium, showing that the immunostaining was specific for CGRP.

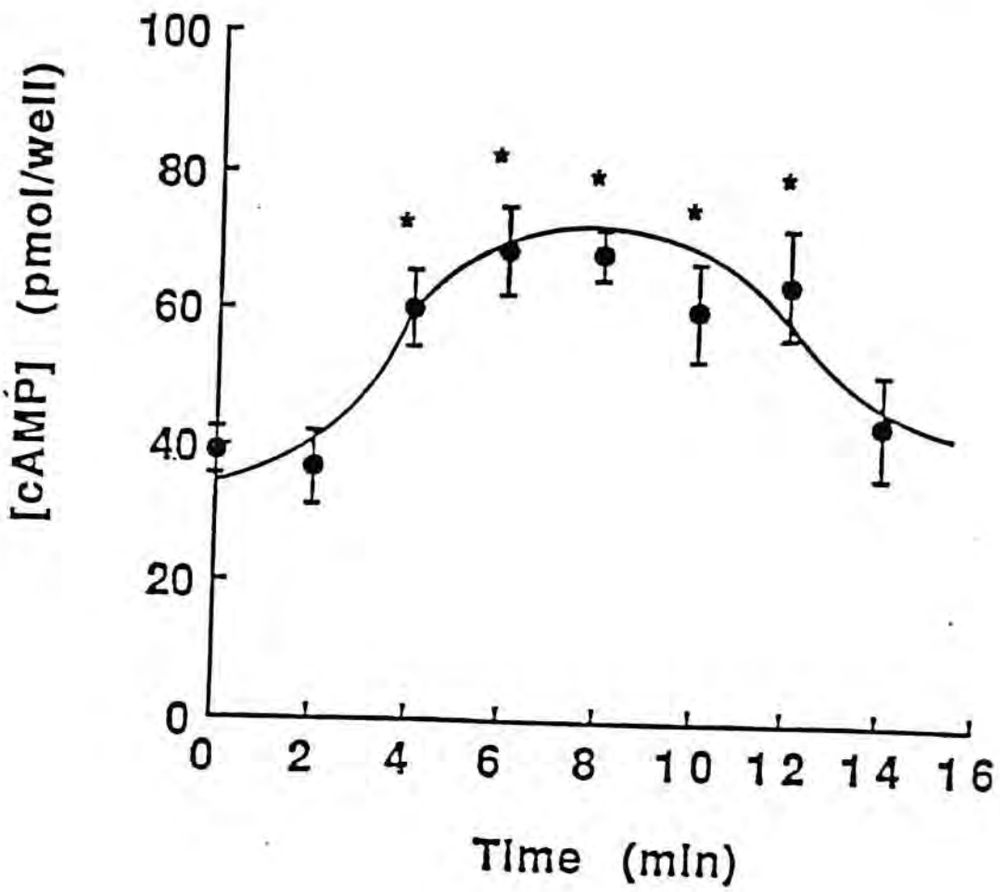


Fig.III.2.6.

Time course of rise of intracellular cAMP concentration in rat epididymal monolayers upon stimulation with CGRP (500 nM). Values are means ± s.e.m. for 9 to 12 cultures.
* Significantly different from values obtained at time zero at $p < 0.05$.

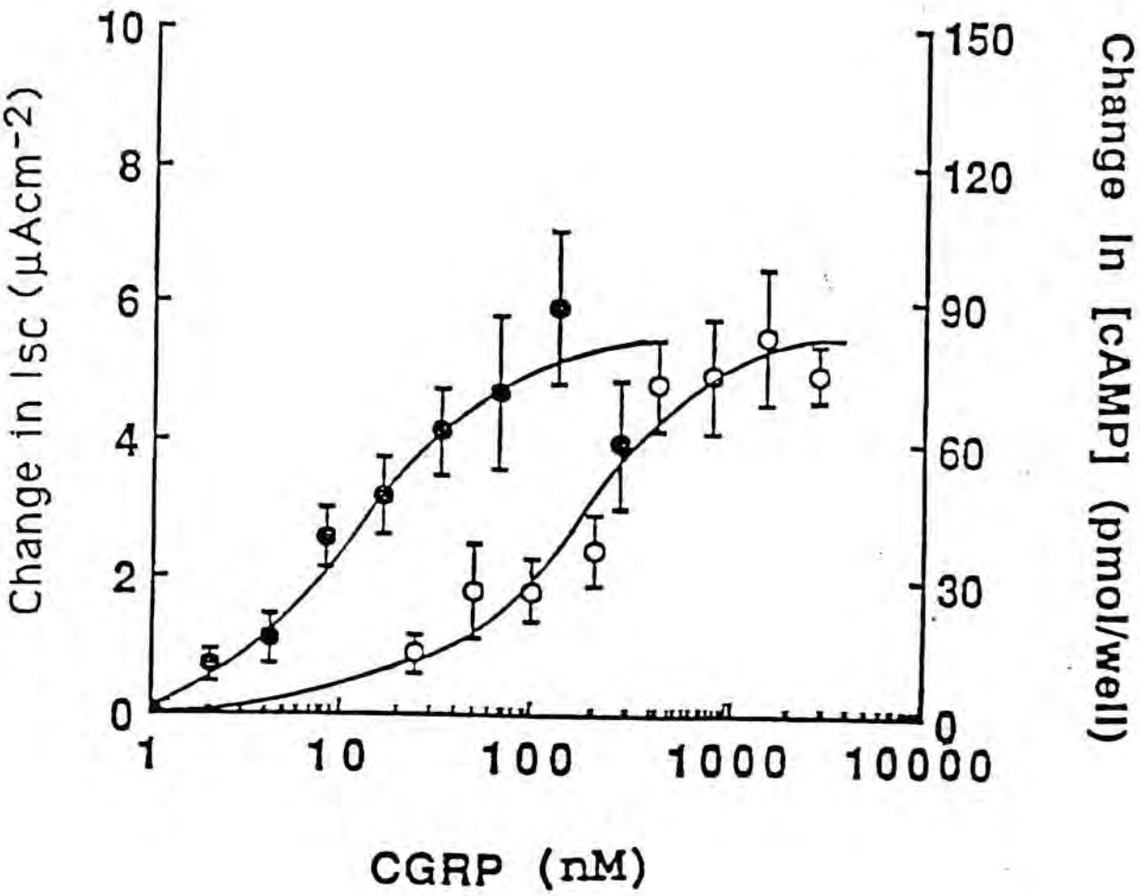
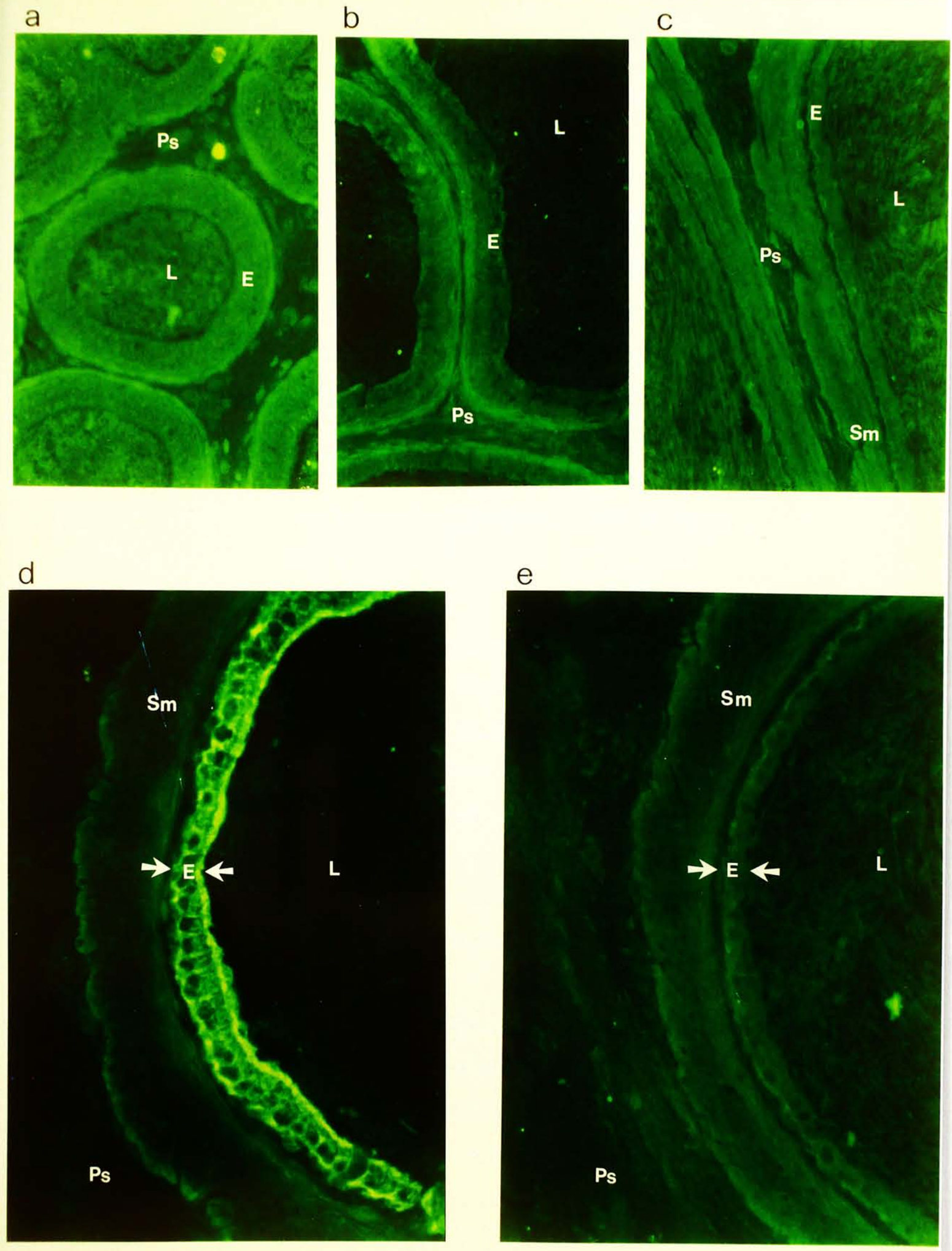


Fig.III.2.7.

Effect of various concentration of CGRP on the accumulation of intracellular cAMP (o) in rat epididymal monolayers. (•) shows the concentration-dependence of CGRP for the stimulation of short-circuit current. Values are means ± s.e.m. for 4 to 12 experiments.

Plate 3

(a), (b) and (c) show respectively the photomicrographs of the intial segment, caput and corpus epididymidis of rat. No specific immunostaining for CGRP could be found in the lumen (L), the epithelium of the epididymal tubules (E) or the peritubular space (Ps) (x100). In (d), a portion of a tubule in the rat cauda epididymis is shown. Intense immunofluorescence was observed in the epithelium (E) as indicated by the white arrows. No immunoreactivity was found in the lumen of the tubule (L), the smooth muscle (Sm) and the peritubular space (Ps) (x150). (e) shows the photomicrograph of a similar section in which the primary antibody was preadsorbed with 40 μM of CGRP before incubation. No immunoreactivity was found in the epithelium, showing that the immunostaining was specific for CGRP (x150).


a
Ps
L
E
b
L
E
Ps
c
E
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Ps
Sm
d
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Ps

Discussion

The present study showed that CGRP stimulated a time- and dose-dependent rise in I_{SC} and $[cAMP]_i$ in cultured rat epididymal epithelium. The I_{SC} response to CGRP had many features in common with that to other peptide hormones. First, the angiotensins (Wong et al., 1990), bradykinin (Cuthbert & Wong, 1986) and endothelin-1 (Wong et al., 1989) elicited a rapid but transient increase in I_{SC} similar to that by CGRP (Fig.III.2.2.i.). Secondly, the receptors for these peptides underwent desensitization upon repeated stimulations. Receptor desensitization for CGRP have been reported in rat myocytes (Fisher et al., 1988) and the very transient nature of the I_{SC} response seen at high concentration (Fig.III.2.2.i.) might be an expression of this desensitization phenomenon (Ono et al., 1989). Receptor desensitization may be important for the regulation of Cl^- secretion by preventing overstimulation of the epithelium in the continuous presence of the peptide.

There was discrepancy between the I_{SC} response and the increase in $[cAMP]_i$ upon CGRP stimulation. The dose-response relationships showed that the EC_{50} for stimulation of cAMP (150nM) is about ten times higher than that of I_{SC} (15 nM). The I_{SC} response reached a maximum at doses that produced only 50% increase in cell cAMP (Fig.III.2.7). Similar observation has been found for adrenaline (Wong & Huang, 1990). The cAMP pool which was responsible for the I_{SC} response might represent only a small fraction of the total cAMP. Thus, an increase in the rate of synthesis or the size of the small fraction could be difficult to detect when the total pool of intracellular cAMP was measured.

The CGRP has been found in nerve fibres in the salivary glands (Ekström et al., 1988), the kidney (Geppetti et al., 1989), the pancreas (Seifert et al., 1985), the colon (Ekblad et al., 1988) as well as the seminal vesicles and the vas deferens (Gibbins et al., 1985). It is widely believed that CGRP is a neuropeptide secreted from sensory nerve

endings. The present study demonstrated that the CGRP was present in the epithelial cells of the rat cauda epididymidis. The peptide might be synthesized *de novo* in the epithelium and act in a paracrine fashion on neighbouring epithelial cells (Plate 3). Immunoreactive CGRP was found exclusively in the cauda epididymidis suggesting that the caudal region of the rat epididymis might be regulated by local factors which were different from those in the proximal regions. This might contribute to the specialized microenvironment in the cauda epididymidis which is vital to storage of spermatozoa.

Chapter III.3.

Characterization of intracellular calcium store using ATP as the calcium mobilizing agonist

Summary

The present study characterized the properties of an ATP-sensitive Ca^{2+} pool in rat epididymal cells. In HEPES-buffered solution containing 2.5 mM free Ca^{2+}, ATP triggered single Ca^{2+} spikes in a dose-dependent fashion. In nominally Ca^{2+}-free solution, the peaks of successive Ca^{2+} spikes diminished upon repeated ATP stimulations. Addition of Sr^{2+} (2.5 mM) to Ca^{2+} free solution after ATP stimulation did not cause changes in fluorescence signals. However, in the presence of Sr^{2+}, ATP gave rise to apparent repetitive 'Ca^{2+} spikes' of similar magnitudes upon repeated stimulations. Increasing the time of exposure in Ca^{2+}-free solution containing 50 μM EGTA rapidly decreased the $[Ca^{2+}]_i$ response to subsequent ATP stimulation. On the other hand, increasing the time of exposure in Sr^{2+} containing solution in Ca^{2+}-depleted cells rapidly increased the apparent $[Ca^{2+}]_i$ response to subsequent ATP stimulation. The results suggested that the agonist-sensitive Ca^{2+} store in the epididymal cells might be rapidly exchanging with the extracellular compartment.

Introduction

It is now known that the stimulus-secretion coupling in response to neurohumoral and local stimuli to the epididymis involves both cAMP and Ca^{2+} as second messengers (Chapter III.1. & III.2). The cAMP cascade involves a coupling G protein, the membrane bound adenylate cyclase and the protein kinase A which binds cAMP and causes phosphorylation of Cl^- channels (Huang et al., 1992b). However, the identity of the internal store from which Ca^{2+} is released has not been known with certainty.

In vivo and *in vitro* studies have shown that Cl^- secretion across the epididymal epithelium could be stimulated by exogenous ATP (Wong, 1988b). In Ehrlich Ascites Tumour Cells (Dubyak & De Young, 1985), ATP has been found to mobilize Ca^{2+} from internal store, leading to an increase in $[Ca^{2+}]_i$. Release of internal Ca^{2+} upon ATP stimulation could be due to the hydrolysis of phophoinositides and the generation of IP_3 (Burnstock, 1990). The present study characterized the internal Ca^{2+} store releasable by ATP.

Methods

The microfluorimetric technique has been described (Chapter II.5.). Composition of normal Ca^{2+} buffered solution and solution for preincubation have been described in Appendix III. Ca^{2+} free solution I contained 50 μM EGTA without any added $CaCl_2$ and the free Ca^{2+} concentration was expected to be less than 1 μM. Ca^{2+} free solution II contained no EGTA and no added $CaCl_2$ and the free Ca^{2+} concentration was estimated to be around 10 to 20 μM. In Sr^{2+} containing solution, $CaCl_2$ was replaced by an equimolar amount of $SrCl_2$. Other constituents of these solutions were identical to those in normal Ca^{2+} solution. ATP was prepared as 1 mM stock dissolved in the particular solution to be used in the experiments.

Results

Fig. III.3.1. shows the effects of increasing doses of ATP on $[Ca^{2+}]_i$ in a single epididymal cell when it was bathed in normal Ca^{2+} solution. The $[Ca^{2+}]_i$ response to ATP manifested as a single Ca^{2+} spike and the peak $[Ca^{2+}]_i$ was dependent on the dose. The inset shows the effects of varying the dose of ATP on the change in $[Ca^{2+}]_i$ defined as the peak $[Ca^{2+}]_i$ response. The dose-response curve exhibited a sigmoidal profile with a threshold dose of 2×10^{-8} M and a EC_{50} of about 5×10^{-7} M. Maximal response was attained at 2×10^{-5} M ATP. Fig. III.3.2. shows the effects of sequential stimulations with ATP and thapsigargin (Tg) on $[Ca^{2+}]_i$ in normal Ca^{2+} solution. In (i), ATP (20 μM) elicited a single Ca^{2+} spike. After $[Ca^{2+}]_i$ has returned to the basal level, ATP was washed out and the cell was stimulated with Tg (4 μM). Another Ca^{2+} spike was observed similar to that elicited by ATP. The cell was again stimulated with ATP (20 μM) but no $[Ca^{2+}]_i$ response was found. (ii) shows the control experiment in which the cell was stimulated successively with ATP (20 μM) without intervening Tg stimulation. The first stimulation with ATP triggered a Ca^{2+} spike and subsequent stimulation gave rise to a second spike similar to the first one in magnitude.

It has been reported that divalent cations had different properties in terms of their permeation across the plasma membrane as well as the ability to replenish the internal Ca^{2+} store (Kwan & Putney, 1990). Studies on these cations might give a clue to the relationships between the internal Ca^{2+} store and the plasma membrane. In particular, the excitation spectrum of fura-2-Sr^{2+} has been shown to be almost identical to that of fura-2-Ca^{2+}, suggesting that changes in fluorescence signals due to Sr^{2+} could be used to estimate changes in intracellular Sr^{2+} concentration ($[Sr^{2+}]_i$). Fig.III.3.3. shows that after the ATP-induced Ca^{2+} spike in Ca^{2+} free solution II (see Methods), addition of Sr^{2+} to a concentration of 2.5 mM did not affect the apparent $[Ca^{2+}]_i$, suggesting that Sr^{2+} did not permeate through the plasma membrane. Subsequent admission of Ca^{2+} (2.5 mM) gave

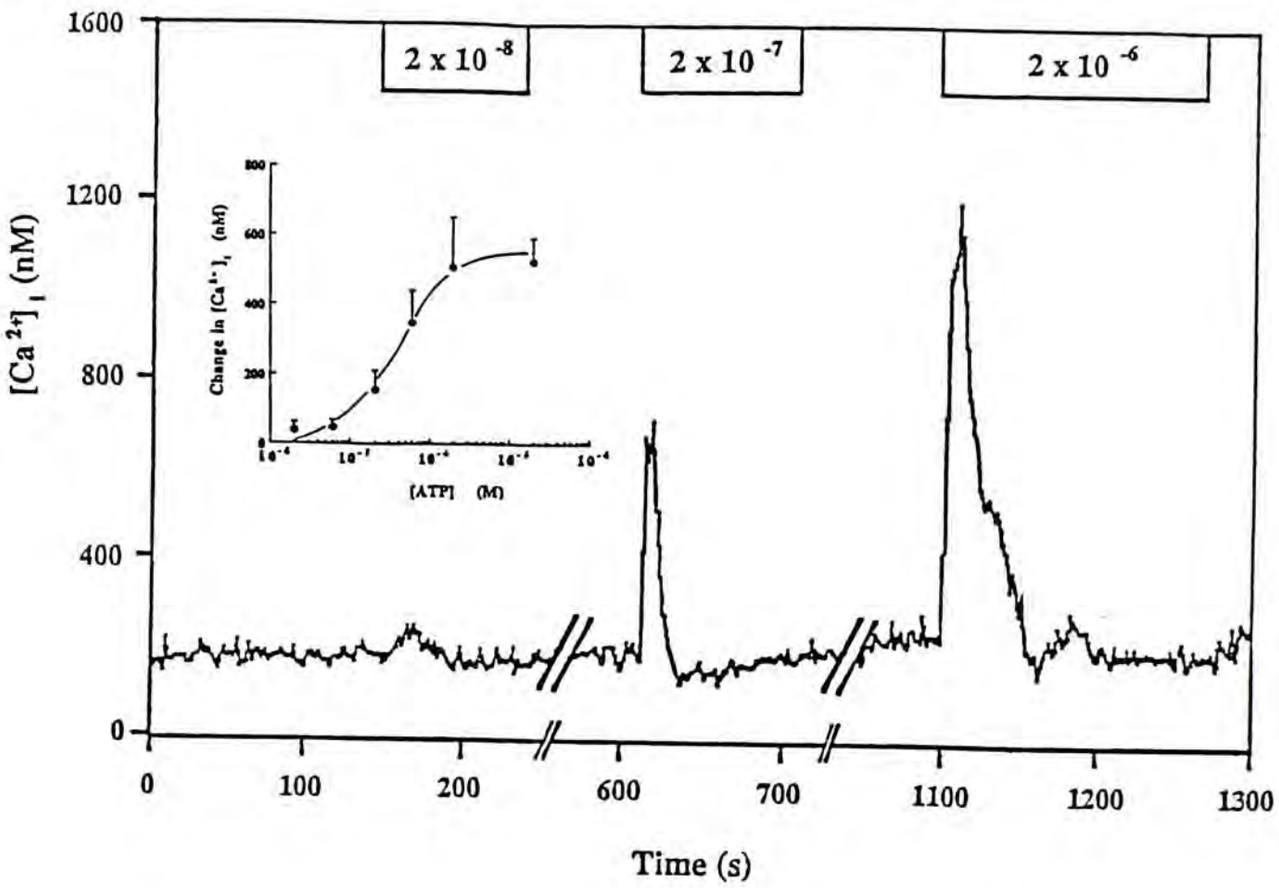

Fig.III.3.1.

Effects of varying the dose of ATP on $[Ca^{2+}]_i$ in a single epididymal cell bathed in normal Ca^{2+} solution. The $[Ca^{2+}]_i$ response to ATP manifested as a single Ca^{2+} spike. ATP was washed out between successive stimulations. The open bars on the top represent the duration of ATP stimulation and the figures therein indicate the dose of ATP in molar (M). The double-slash lines between successive ATP stimulations represent a recovery period of several minutes after each stimulation. The inset shows the dose-response curve of ATP on $[Ca^{2+}]_i$. The change in $[Ca^{2+}]_i$ was defined as the height of Ca^{2+} spike. Each data point represents the mean ± SE of 6 to 27 experiments.

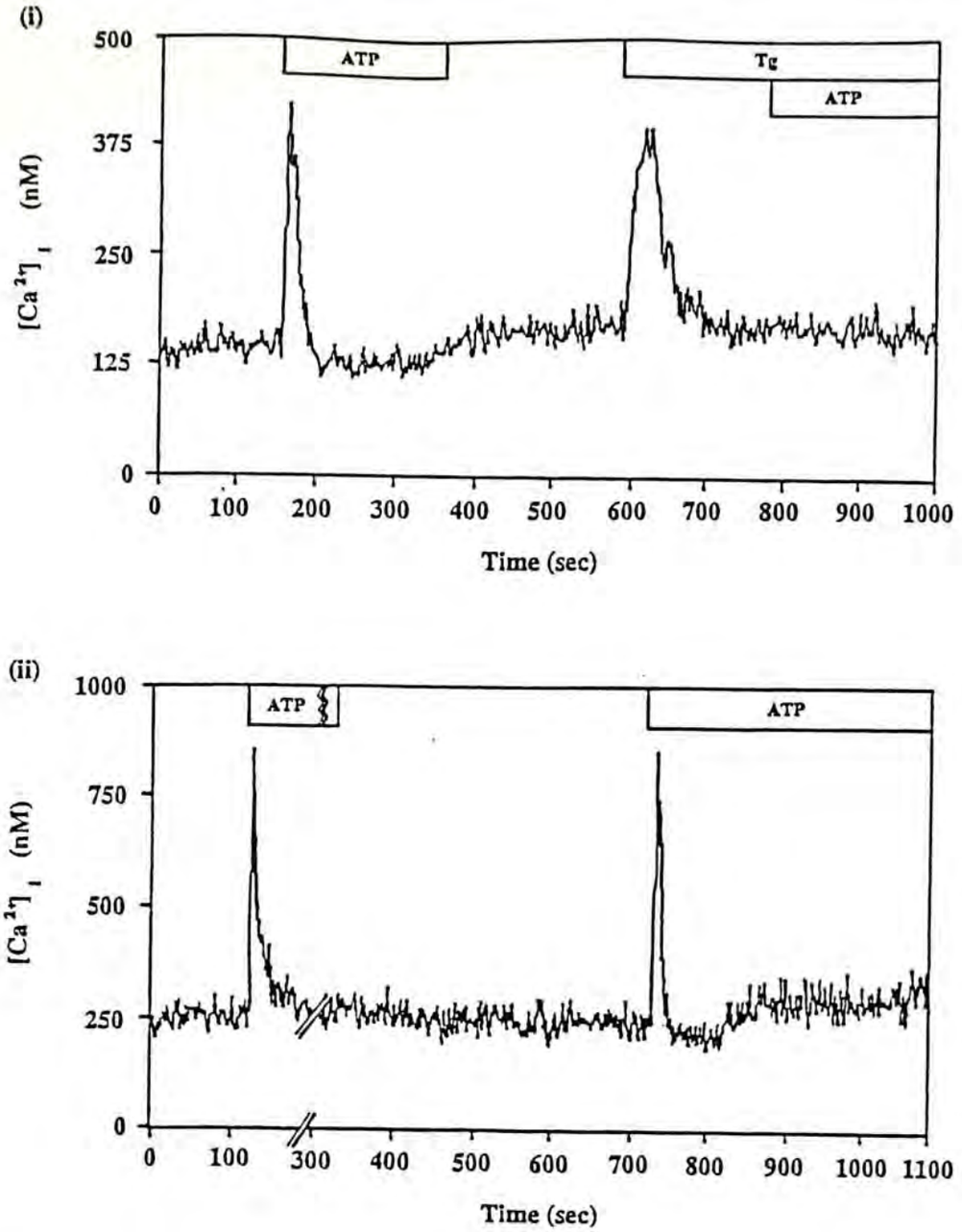

Fig.III.3.2

Effects of sequential stimulation with ATP (20 μM) and Tg (4 μM) on $[Ca^{2+}]_i$ in normal Ca^{2+} solution. The open bars on the top of each panel represent the duration of ATP/Tg stimulation. (i) Tg added after the first ATP stimulation elicited a Ca^{2+} spike but abolished the $[Ca^{2+}]_i$ response to subsequent ATP stimulation. (ii) Successive ATP stimulations without intervening Tg stimulation elicited Ca^{2+} spikes of similar magnitudes. The double-slash lines indicate a period of about 2 minutes. Each tracing is representative of at least four separate experiments.

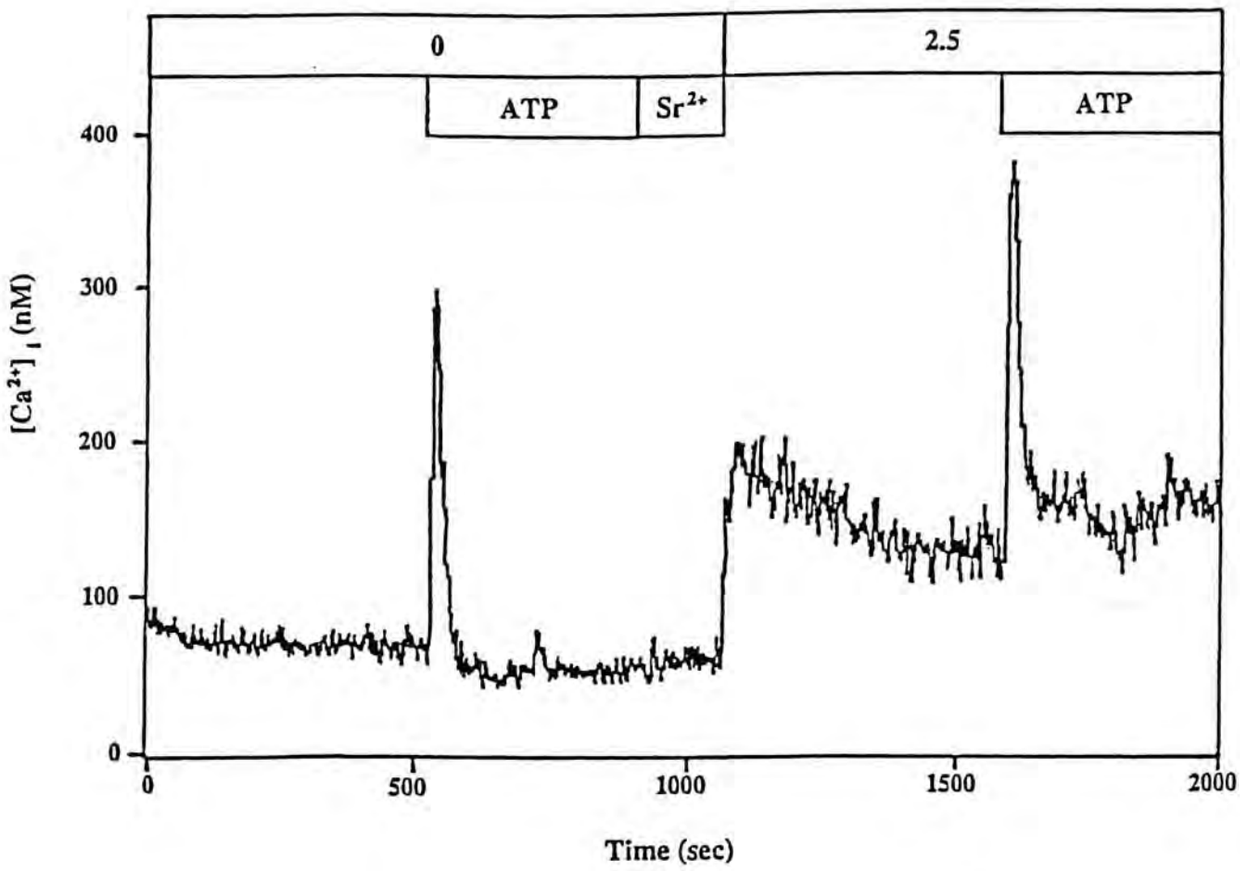

Fig.III.3.3

Effects of Sr^{2+} on the apparent $[Ca^{2+}]_i$ after ATP stimulation. The values on top of the figure indicate extracellular Ca^{2+} concentration in millimolar (mM). The open bars underneath represent the duration at which ATP (20 μM) or Sr^{2+} (2.5 mM) was added. After ATP stimulation in Ca^{2+} free solution II (see Materials and Methods), addition of Sr^{2+} (2.5 mM) did not cause changes in fluorescence signal. Addition of Ca^{2+} (2.5 mM) led to an increase in $[Ca^{2+}]_i$ and stimulation with ATP thereafter elicited another Ca^{2+} spike. The record was representative of 4 separate experiments.

rise to an increase in $[Ca^{2+}]_i$ and stimulation with ATP thereafter elicited another Ca^{2+} spike. Fig.III.3.4.i shows the effects of repeated stimulations with ATP in Ca^{2+} free solution II. The magnitudes of successive Ca^{2+} spikes followed a decremental pattern upon repeated ATP stimulations. ii shows a similar experiment in the presence of Sr^{2+} (2.5 mM). It can be seen that the apparent $[Ca^{2+}]_i$ responses to ATP did not diminish upon repeated stimulations. Therefore, Sr^{2+} might be able to replenish the internal Ca^{2+} store between successive ATP stimulations without having to permeate through the plasma membrane (see fig.III.3.3). This led to the speculation that epididymal cells might possess an internal Ca^{2+} store which was in direct continuity with the extracellular compartment. Fig.III.3.5. shows the relationships between the depletion and repletion time and the peak $[Ca^{2+}]_i$ response to ATP. Increasing the depletion time by increasing the duration of exposure in Ca^{2+}-free solution I decreased the $[Ca^{2+}]_i$ response to subsequent ATP stimulation. Depletion was complete after 20 minutes of exposure to Ca^{2+} free solution. On the other hand, increasing the repletion time was found to increase the apparent $[Ca^{2+}]_i$ response to ATP. The internal Ca^{2+} store was first depleted by stimulating the cell with maximal dose of ATP (20 μM) in Ca^{2+} free solution I. It was then exposed to Sr^{2+} (2.5 mM) for various periods of time (repletion time) before ATP stimulation. The apparent $[Ca^{2+}]_i$ response rose rapidly when the repletion time increased from 0 to 2 minutes and then more slowly as repletion time increased from 2 to 20 minutes.

Discussion

Extracellular nucleotides are involved in many physiological processes like platelet aggregation, mast cell degranulation, regulation of local blood flow as well as neuromodulation (Gordon, 1986). Recent evidence has shown that they might affect Cl^- secretion in exocrine tissues (Burnstock, 1990). The effects of ATP could be due to activation of P_2-purinoceptors (Gallacher, 1982) which caused a series of biochemical events, culminating in the final secretory response. Epididymal epithelium has been

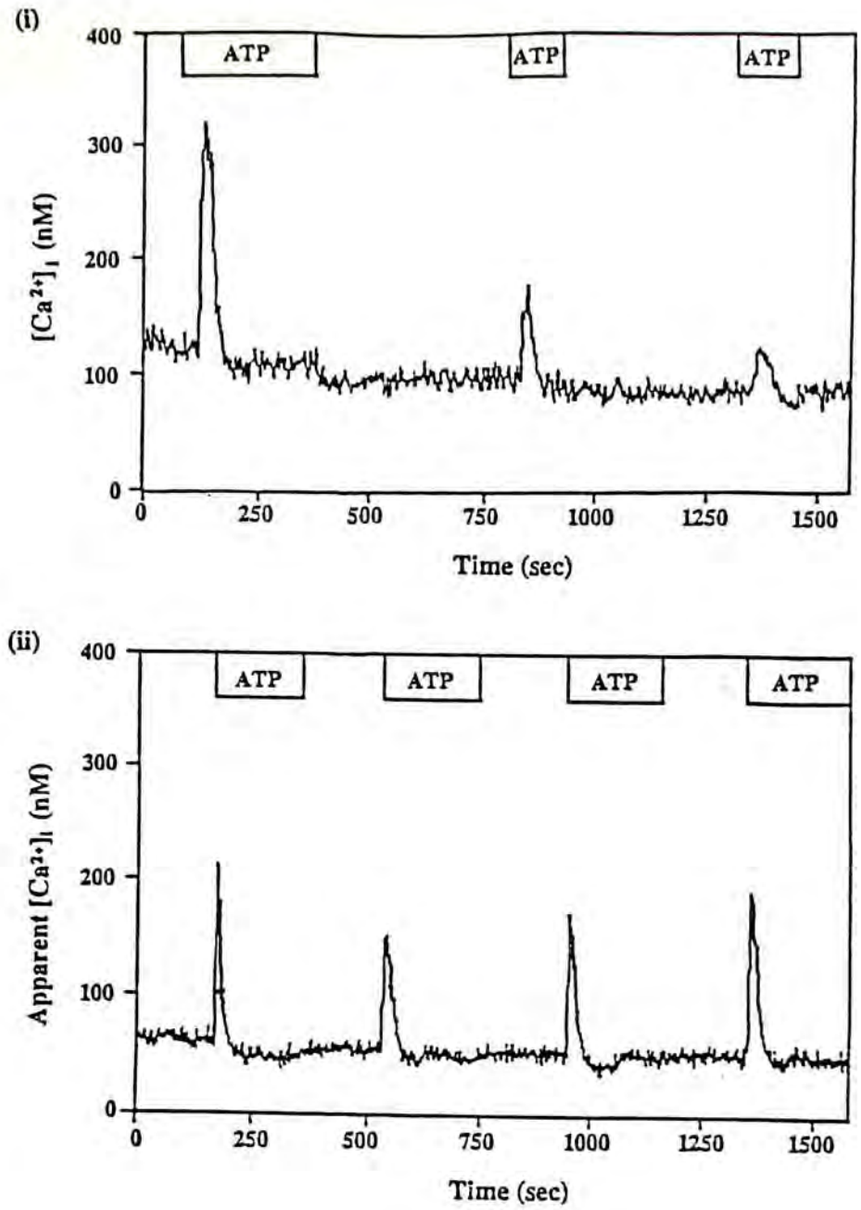

Fig.III.3.4.

Effects of repeated ATP stimulations in Ca^{2+} free solution II and Sr^{2+} containing solution (see Materials and Methods). ATP was washed out between successive stimulations and the open bars on the top of each panel represent the duration of ATP (20 μM) stimulation. (i) In Ca^{2+} free solution, the magnitudes of Ca^{2+} spikes diminished upon repeated stimulations. (ii) In Sr^{2+} containing solution, ATP gave rise to apparent repetitive 'Ca^{2+} spikes' of similar magnitudes upon repeated stimulations. Each of the record is representative of 4 separate experiments.

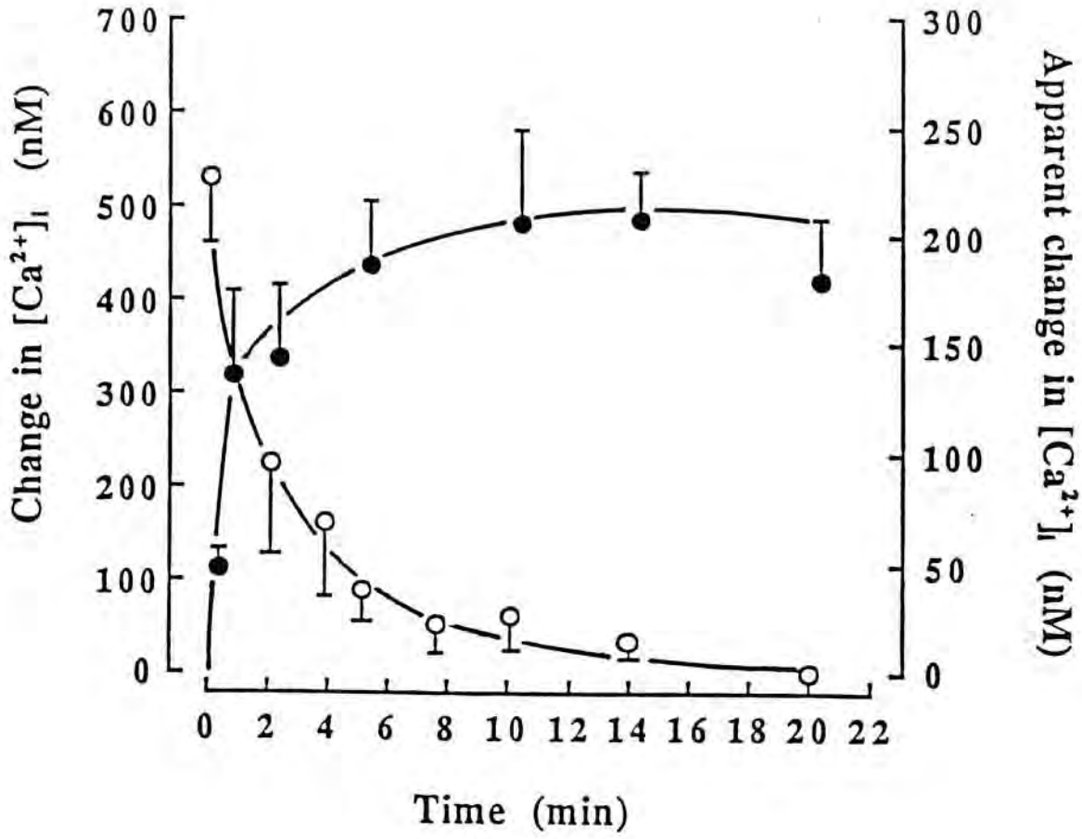

Fig.III.3.5.

Relationships between the depletion and repletion time and the $[Ca^{2+}]_i$ response to subsequent ATP stimulation. Increasing the time of exposure in Ca^{2+} free solution I (See Methods) (depletion time) decreased the $[Ca^{2+}]_i$ response to subsequent ATP stimulation (open circles). Because of the time required for experimental manipulations and for $[Ca^{2+}]_i$ to reach a stable level when the bathing solution was changed from normal to Ca^{2+}-free solution, the shortest depletion time at which the $[Ca^{2+}]_i$ response could be recorded accurately was 2 minutes. At 0 time, depletion of the Ca^{2+} store has not commenced, therefore, the $[Ca^{2+}]_i$ response at that time was obtained when the cell was stimulated with ATP in normal Ca^{2+} solution before exposure to Ca^{2+} free solution. In another set of experiments, the epididymal cells were Ca^{2+} depleted by stimulating with maximal dose of ATP in Ca^{2+} free solution I. They were then exposed to Sr^{2+} containing solution for various periods of time (repletion time) before ATP stimulation (closed circles). At 0 time the repletion process has not commenced and the apparent $[Ca^{2+}]_i$ response was therefore zero. The apparent $[Ca^{2+}]_i$ response rose rapidly when the repletion time increased from 0 to2 minutes and more slowly when the repletion time increased from 2 to 20 minutes. Each data point is mean ± SE of 4 to 27 separate experiments.

shown to possess P_2-purinoceptors on the apical aspect of the cells (Wong, 1988b) and the secretory response to ATP might be attributed to Ca^{2+} release from an agonist-sensitive store as reported in Ehrlich Ascites Tumour Cells (Dubyak & De Young, 1985). The evidence came from the observation that thapsigargin (Chapter III.1.) abolished the $[Ca^{2+}]_i$ response to ATP (see Fig III.3.2.). Although the phosphoinositides turnover has not been measured in the present study, circumstantial evidence has shown that the release of internal Ca^{2+} upon ATP stimulation could be due to the hydrolysis of phosphoinositides and the generation of IP_3 (Burnstock, 1990). IP_3 might act on specific receptors on internal store, causing release of Ca^{2+} into the cytosol (Berridge & Irvine, 1989).

It has been shown that the excitation spectrum of fura-2-Sr^{2+} was almost identical to that of fura-2-Ca^{2+} (Kwan & Putney, 1990). Alteration in cytosolic Sr^{2+} concentration might elicit changes in fluorescence signals at least qualitatively similar to that of Ca^{2+}. The present study showed that after ATP stimulation in Ca^{2+} free solution, addition of Sr^{2+} to a final concentration of 2.5 mM did not cause changes in fluorescence signals (Fig.III.3.3). This suggested that the plasma membrane of epididymal cells was not permeable to Sr^{2+} even after ATP stimulation (see below). The result was in contrast to those in lacrimal acinar cells (Kwan & Putney, 1990) where an increase in Sr^{2+} influx across the plasma membrane was observed after methacholine stimulation. However, it was also found that extracellular Sr^{2+} could replenish the Ca^{2+} store as shown by the apparent repetitive 'Ca^{2+} spikes' upon repeated ATP stimulations (Fig.III.3.4). It is known that without EGTA, the free Ca^{2+} concentration in distilled water could be as high as 10 - 20 μM. To show that the apparent repetitive 'Ca^{2+} spikes' in Sr^{2+}-containing solution were not due to replenishment of Ca^{2+} store by trace amount of contaminant Ca^{2+} present in the solution, simple omission of extracellular Ca^{2+} (Ca^{2+} free solution II) was found to cause diminishing Ca^{2+} responses to repeated ATP stimulations. Therefore, the apparent repetitive 'Ca^{2+} spikes' might be attributed to the replenishment of Ca^{2+} store by

extracellular Sr^{2+} in the bathing solution. The finding that Sr^{2+} did not permeate through the plasma membrane but could replenish the internal store suggested the existence of a Ca^{2+} store which was in direct continuity with the extracellular compartment (Casteels & Droogmans, 1981). Studies on electron microscopy have demonstrated the existence of Sr^{2+} containing vesicles on the cytosolic aspect of the plasma membrane in vascular smooth muscle cells (Somlyo & Somlyo, 1971). It was observed that the ATP-sensitive Ca^{2+} pool in the epididymal cells could be depleted very rapidly in Ca^{2+}-free solution (Fig.III.3.5). Depletion was complete after 20 minutes of exposure to Ca^{2+}-free solution. Using Sr^{2+} as a Ca^{2+} substituent, it was also found that the pool could be repleted very rapidly. Sr^{2+} was used because at a concentration of 2.5 mM, it did not permeate through the plasma membrane (see above), avoiding the complication due to Sr^{2+} uptake into the store from the cytosol. The apparent $[Ca^{2+}]_i$ response to ATP rose rapidly at short repletion period (0 to 2 min) and more slowly at long period of repletion (2 to 20 min). It could be that the repletion process exhibited saturation kinetics such that initially the rate of repletion was high but as increasing portion of the Ca^{2+} pool has been repleted, the rate of repletion became lower.

It is generally believed that the filling state of the Ca^{2+} store can affect Ca^{2+} influx across the plasma membrane (Putney, 1986). The present study demonstrated that depletion of the store by stimulating the cell with maximal dose of ATP in Ca^{2+} free solution triggered Ca^{2+} influx which was selective against Sr^{2+} (see fig.III.3.3). This was different from that which led to direct replenishment of the Ca^{2+} store, which could not discriminate between Sr^{2+} and Ca^{2+} (see above). Therefore in epididymal cells, the mechanisms by which Ca^{2+} entry was activated upon depletion of the store could not be explained entirely on the basis of chemical gradient as proposed by Putney (1986). The 'crosstalk' between the plasma membrane and the internal store might involve more subtle

electrical changes over the plasma membrane or the generation of second messengers in the cytosol. These possibilities await further investigation.

Chapter III.4

Ca^{2+} handling mechanisms in single cultured rat epididymal cells.

Summary

The present study investigated the regulation of intracellular Ca^{2+} homeostasis in single rat epididymal cells. In HEPES-buffered solution containing either 2.5 mM Ca^{2+} (normal solution), 0 mM Ca^{2+} plus 50 μM EGTA (Ca^{2+} free solution) or 2.5 mM Ca^{2+} plus 1 mM La^{3+} (La^{3+} containing solution), Tg elicited a transient rise in $[Ca^{2+}]_i$. The peak rise in $[Ca^{2+}]_i$ upon Tg stimulation was not affected in Ca^{2+} free solution but was significantly increased in the presence of La^{3+} (1 mM). When La^{3+} (6 mM) was added during the declining phase of the Tg-induced transient in Na^+-free solution, an additional but transient rise in $[Ca^{2+}]_i$ was observed.

When the cells were bathed in Ca^{2+} free solution, addition of 2.5 mM Ca^{2+} increased $[Ca^{2+}]_i$ by 61.4 ± 10.6 nM. Ca^{2+} was washed out and the cells were stimulated with Tg. After the transient $[Ca^{2+}]_i$ rise, repletion of Ca^{2+} gave rise to an increase in $[Ca^{2+}]_i$ by 196.2 ± 32.7 nM which was significantly greater than that before Tg stimulation. In normal solution, removing ambient Ca^{2+} after the Tg-induced $[Ca^{2+}]_i$ transient decreased $[Ca^{2+}]_i$ to a value below the prestimulated $[Ca^{2+}]_i$. Addition of 2.5 mM Sr^{2+} had no effect on $[Sr^{2+}]_i$. Replacing Sr^{2+} by Ca^{2+} thereafter led to a rise in $[Ca^{2+}]_i$ above the prestimulated $[Ca^{2+}]_i$. The results suggested that in epididymal cells, Ca^{2+} was constantly released from an intracellular store and was sequestered into the store by a Tg-sensitive Ca^{2+}ATPase. When the latter was inhibited, Ca^{2+} was partially extruded out of the cells by a La^{3+}-sensitive Ca^{2+}ATPase on the plasma membrane. Depletion of the intracellular Ca^{2+} store activated a Ca^{2+} entry pathway which was selective against Sr^{2+}.

Introduction

Upon stimulation with Ca^{2+} mobilizing agonists, intracellular Ca^{2+} concentration increases due to Ca^{2+} release from internal store and Ca^{2+} influx into the cells (Chapter III.1.& III.3.). The increase in $[Ca^{2+}]_i$ must be regulated to ensure the maintenance of a low $[Ca^{2+}]_i$. The present study investigated the regulation of intracellular Ca^{2+} homeostasis in single rat epididymal cells.

Methods

The composition of normal Ca^{2+} buffered solution has been described in appendix III. In La^{3+}-containing solution, $MgSO_4$ was replaced by $MgCl_2$ and Na_2HPO_4 was omitted. In Sr^{2+} containing solution, $CaCl_2$ was replaced by equimolar amount of $SrCl_2$. In Na^+ free solution, NaCl was replaced by NMDGCl and NaHEPES by TrisHEPES titrated to pH 7.4.

Results

Fig.III.4.1. shows the effects of thapsigargin (Tg) on $[Ca^{2+}]_i$ in 3 single epididymal cells. i) shows that in HEPES-buffered solution containing 2.5 mM free Ca^{2+}, Tg led to a transient rise in $[Ca^{2+}]_i$. ii) shows the result of a similar experiment in Ca^{2+} free solution containing 50 μM EGTA. The magnitude of response was almost identical to that in the presence of Ca^{2+} but the response was more transient. iii) shows the Ca^{2+} response to Tg in normal solution containing 2.5 mM Ca^{2+} plus 1 mM La^{3+}. The latter has been widely used as a Ca^{2+} antagonist to block both Ca^{2+} influx into and Ca^{2+} efflux out of the cells. In the presence of La^{3+}, the Ca^{2+} response to Tg was enhanced and the declining phase of the Ca^{2+} transient was slower than that in normal Ca^{2+} solution. The inset shows the average results of these experiments. The mean Ca^{2+} respone to Tg in HEPES-buffered Ca^{2+}-containing solution was 260.9 ± 45.9 % over the basal $[Ca^{2+}]_i$; that in Ca^{2+} free solution was 389.0 ± 116.3 % ($P > 0.05$) whereas in La^{3+} containing

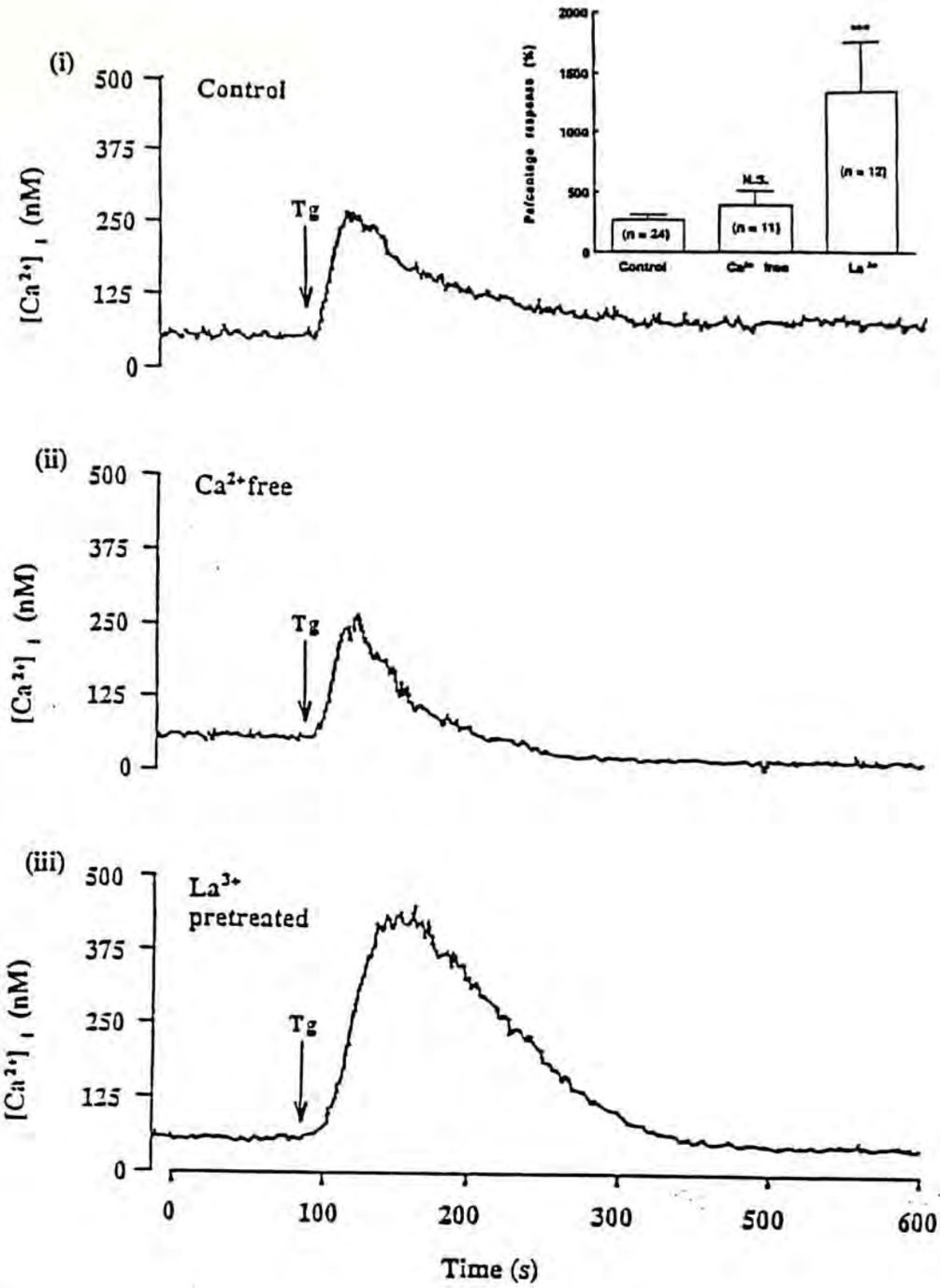

Fig.III.4.1.

Effects of thapsigargin (Tg, 2 μM) on $[Ca^{2+}]_i$ in single epididymal cells when they were bathed in HEPES-buffered solution containing 2.5 mM free Ca^{2+} (i), in Ca^{2+}-free solution containing 50 μM EGTA (ii) and in Ca^{2+}-containing solution (2.5 mM) containing 1 mM La^{3+}. The inset shows the average values of these experiments. Each error bar represents on S.E.M. (*** $P < 0.001$, NS no significance. Students's unpaired t-test).

solution, the mean value was 1350.0 ± 417.4 % ($P < 0.001$). The transient nature of the $[Ca^{2+}]_i$ response to Tg suggested that some processes were at work to lower $[Ca^{2+}]_i$ to the basal level. It could be that at 1mM, La^{3+} did not completely abolish the Ca^{2+}ATPase on the plasma membrane or Ca^{2+} was being extruded out of the cell by a La^{3+}-insensitive mechanism, notably the Na^+/Ca^{2+} exchange (Gill et al., 1984). It was also possible that cytosolic Ca^{2+} was resequestered into a Ca^{2+} store by a Tg-insensitive mechanism. Fig.III.4.2. shows the effects of high concentration of La^{3+} (6 mM) on the $[Ca^{2+}]_i$ response to Tg in Na^+-free solution (see appendix III). Omission of Na^+ in the bathing solution excluded the possible involvement of the Na^+/Ca^{2+} exchange. Addition of La^{3+} (6 mM) about 1 minute after the peak $[Ca^{2+}]_i$ response to Tg elicited a further but transient rise in $[Ca^{2+}]_i$. When $[Ca^{2+}]_i$ has almost returned to the basal level after Tg stimulation, however, La^{3+} did not not affect $[Ca^{2+}]_i$ to any extent.

In addition to releasing Ca^{2+} from internal store, many Ca^{2+} mobilizing agonists are capable of triggering Ca^{2+} influx into the cells. It is believed that the filling state of the internal store might have an effect on Ca^{2+} influx across the plasma membrane (Putney, 1986). Fig.III.4.3. shows the effects of internal Ca^{2+} store depletion on Ca^{2+} influx. Under basal condition, replenishing extracellular Ca^{2+} from 0 to 2.5 mM increased $[Ca^{2+}]_i$ slightly. Upon subsequent removal of ambient Ca^{2+}, $[Ca^{2+}]_i$ returned to the original level. In this Ca^{2+} free medium, Tg led to a substantial rise in $[Ca^{2+}]_i$. After the Ca^{2+} transient, readmission of extracellular Ca^{2+} triggered a considerable rise in $[Ca^{2+}]_i$. The average values were shown in the inset. Ca^{2+} influx was defined as the peak rise in $[Ca^{2+}]_i$ upon extracellular Ca^{2+} admission to Ca^{2+}-free solution. The basal influx was 61.4 ± 10.6 nM and the Ca^{2+} influx after Tg stimulation was 196.2 ± 32.7 nM. The activated Ca^{2+} influx was significantly greater than the basal influx (Student's paired t-test, $P < 0.01$).

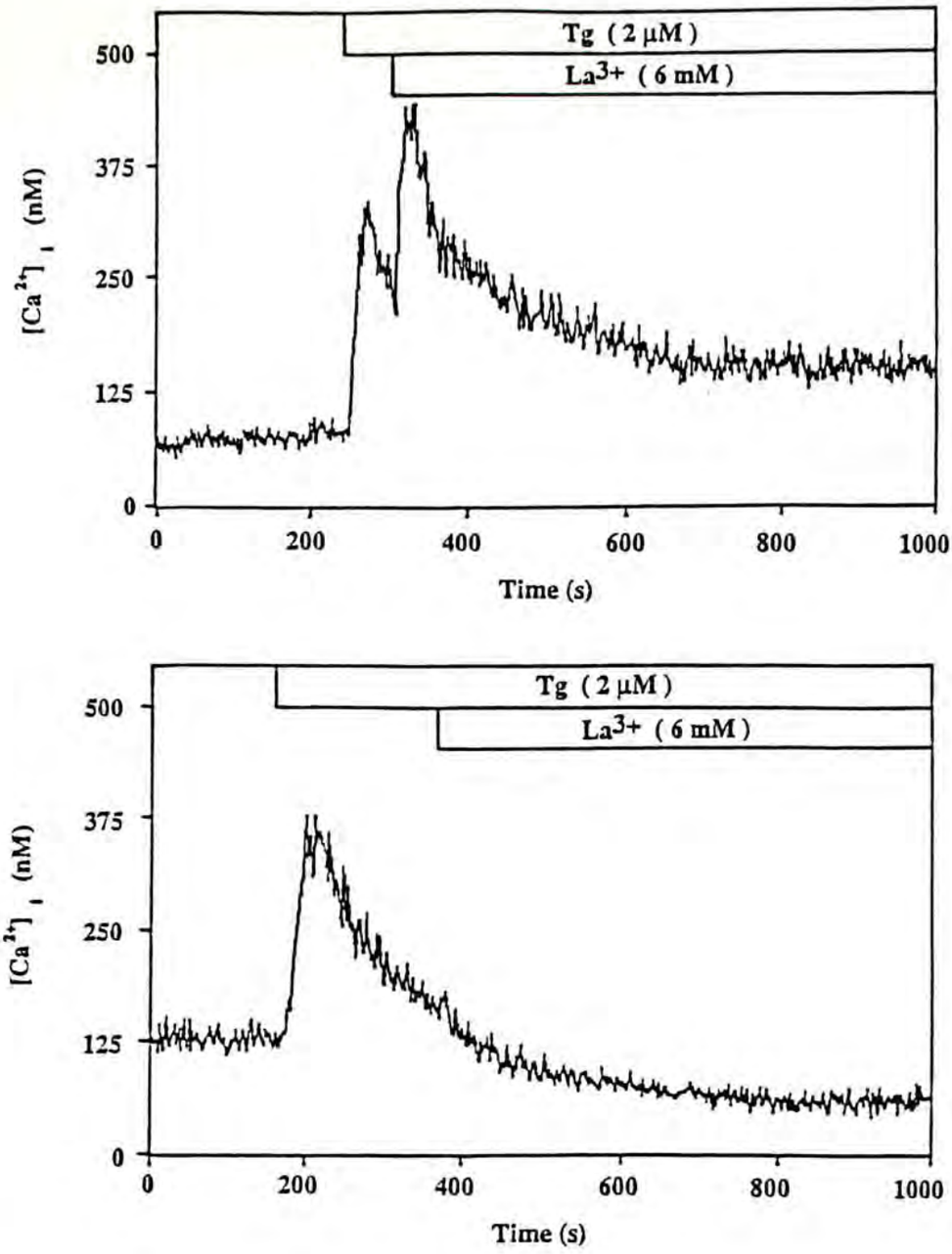

Fig.III.4.2

Effects of high La^{3+} concentration (6 mM) on the $[Ca^{2+}]_i$ response to Tg in Na^+-free solution in single epididymal cells. NaCl was replaced by NMDGCl and NaHEPES by TrisHEPES titrated to pH 7.4. The bars on top of the records indicate the duration of exposure to thapsigargin (Tg, 2 μM) or La^{3+} (6 mM). Each record is representative of 3 separate experiments.

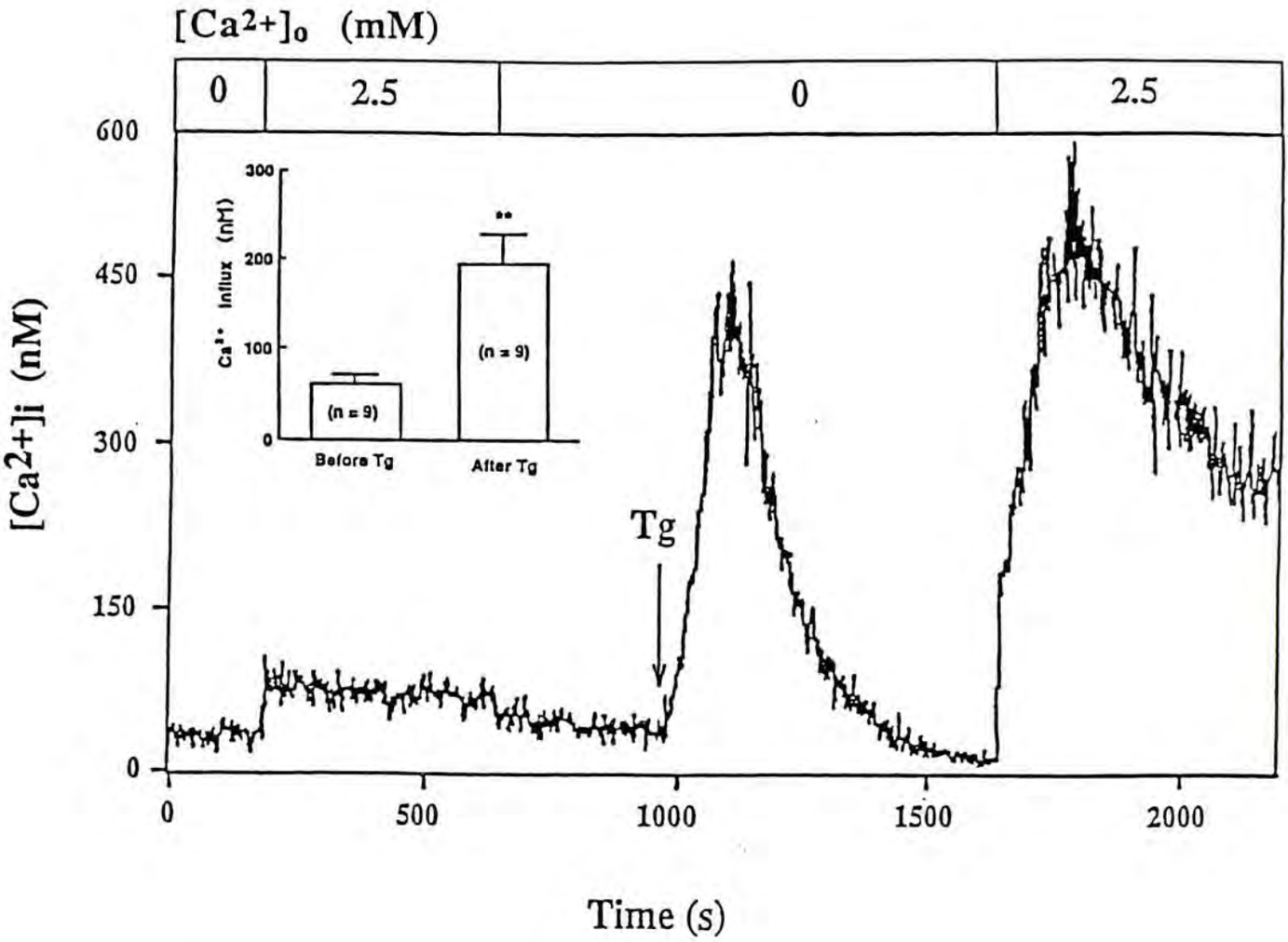

Fig.III.4.3.

Effects of intracellular Ca^{2+} store depletion by thapsigargin on Ca^{2+} influx. The arrow indicates the time when Tg (2 μM) was added. The values on top of the record indicate the extracellular Ca^{2+} concentration (mM).

Fig.III.4.4. shows the results of an experiment demonstrating the selectivity of the Ca^{2+} influx. When bathed in Ca^{2+} containing solution, Tg elicited a transient rise in $[Ca^{2+}]_i$. Removal of ambient Ca^{2+} decreased $[Ca^{2+}]_i$ to a low value. Addition of 2.5 mM Sr^{2+} did not cause changes in fluorescence signals suggesting that Sr^{2+} could not permeate through the plasma membrane. Subsequent admission of extracellular Ca^{2+} led to a substantial rise in $[Ca^{2+}]_i$.

Discussion

The present study shows that free Ca^{2+} in the internal store and the cytosol were in dynamic equilibrium. Under basal condition, Ca^{2+} was constantly released from the internal store and was re-sequestered through the Tg-sensitive Ca^{2+}ATPase. When the latter was inhibited, part of the Ca^{2+} released from the store was extruded out of the cell through a La^{3+}-sensitive Ca^{2+}ATPase on the plasma membrane. The observation that in Na^{+}-free solution, La^{3+} (6mM) added after the peak $[Ca^{2+}]_i$ response to Tg elicited an additional but transient rise in $[Ca^{2+}]_i$, suggesting that the plasma membrane Ca^{2+}ATPase only played a partial role in Ca^{2+} extrusion and the rest of the Ca^{2+} might be re-sequestered into a Ca^{2+} store which was insensitive to Tg. When La^{3+} (6mM) was added after $[Ca^{2+}]_i$ has almost returned to the basal level, $[Ca^{2+}]_i$ was not affected, suggesting that activation of the plasma membrane Ca^{2+}ATPase was a transient phenomenon.

The results also demonstrated that in epididymal cells, depletion of intracellular Ca^{2+} store by Tg activated Ca^{2+} influx across the plasma membrane (Fig.III.4.3.). The influx pathway was selective for Ca^{2+} but impermeable to Sr^{2+} (Fig. III.4.4.). Similar observation has been found when the internal Ca^{2+} store was depleted by extracellular ATP (Chapter III.3). However, the mechanisms by which the internal Ca^{2+} store transmit a signal to the plasma membrane have not been proven with certainty.

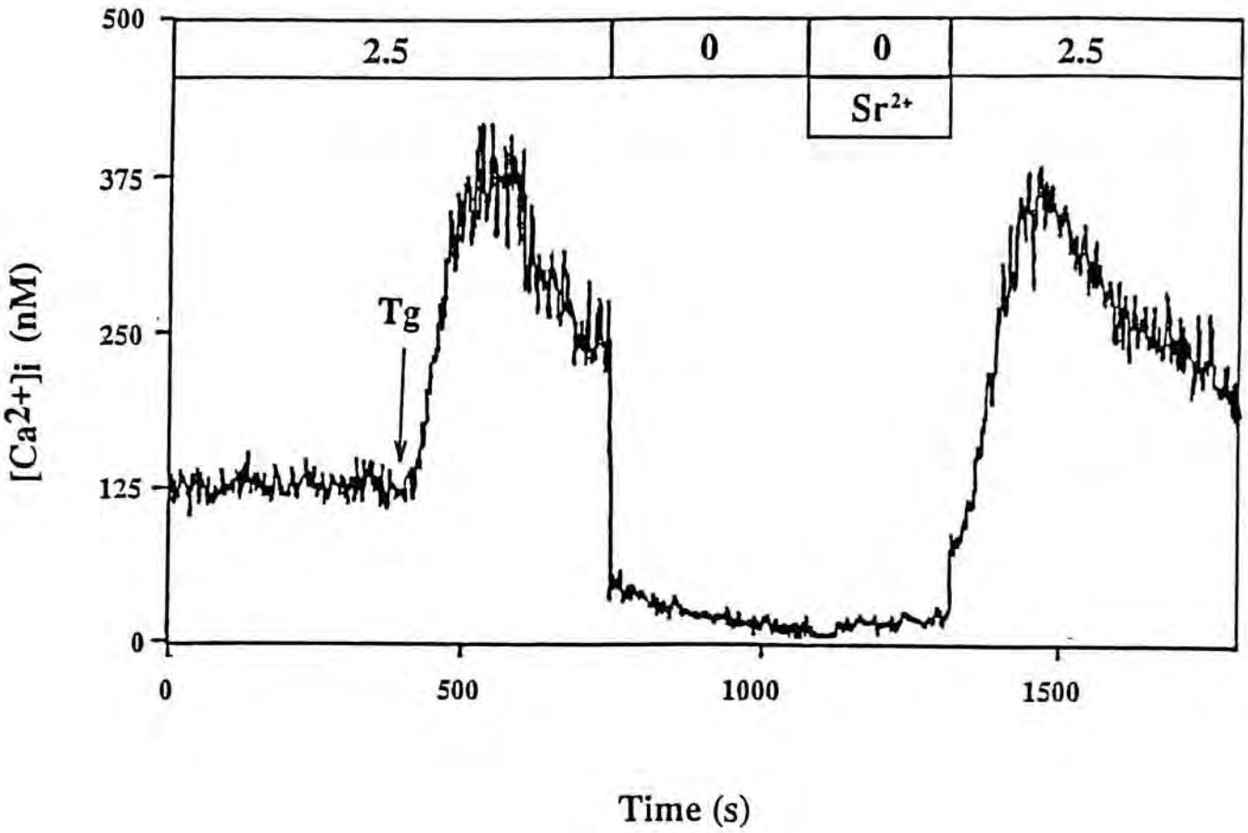

Fig.III.4.4.

Selectivity of the Ca^{2+} influx activated by thapsigargin (Tg) towards Sr^{2+} in signal epididymal cells. The arrow indicates the time when Tg (2 μM) was added. The values on top of the record indicates the extracellular Ca^{2+} concentration (mM). Sr^{2+} on top indicates the duration of exposure to extracellular Sr^{2+} (2.5 mM). The record is representative of three separate experiments.

Chapter III.5

Studies on the effector process in the stimulus-secretion coupling - Characterization of apical Cl^- conductance in cultured rat cauda epididymal cells

Summary

The present study characterized the final effector in the stimuls-secretion coupling i.e. apical Cl^- conductance in cultured rat cauda epididymal epithelium. In HEPES-buffered solution, adrenaline added basolaterally increased I_{SC} and transepithelial conductance (G_t). Decreasing apical Cl^- concentration ($[Cl^-]_a$) progressively by substituting with gluconate increased the I_{SC} response (ΔI_{SC}). The latter could be blocked by the apical application of a Cl^- channel blocker diphenylamine-2-carboxylate (DPC). By substituting apical Cl^- with various anions and measuring the change in I_{SC} upon adrenaline stimulation, the selectivity sequence of the apical anion conductance was found to be $NO_3^- \sim Br^- > Cl^- > I^-$ > gluconate > isethionate. When the tissues were clamped at various transepithelial potential differences (PD_t) from +30 mV (lumen positive) to -30 mV (lumen negative), the relationship between the clamping current response to adrenaline (ΔI_{CL}) and the PD_t applied was linear. The response of the transepithelial conductance to adrenaline (ΔG_t) did not depend on the PD_t applied but was reduced with decreasing apical Cl^- concentration. It is suggested that Cl^- exit across the apical anion conductance is activated in response to neurohumoral or local stimuli, leading to a rise in transepithelial Cl^-secretion.

Introduction

It has been shown that the electrogenic Cl^- secretion in the rat cauda epididymidis is subject to neural, humoral as well as local regulations (Chapter III.1 &2). These factors act on surface receptors of epithelial cells, eliciting a rise in $[Ca^{2+}]_i$ (e.g. a-adrenergic stimulation, ATP) or $[cAMP]_i$ (e.g. ß-adrenergic stimulation, CGRP). The present study investigated the final secretory process in the stimulus-secretion coupling by looking at the properties of apical Cl^- conductance using the short-circuit current technique.

Methods

The techniques of tissue culture and the short-circuit current measurement have been described (Chapter II.1 & II.2). When the apical Cl^- concentration ($[Cl^-]_a$) was reduced, the non-specific effects associated with unilateral ion replacement have to be corrected. Before each experiment, a dummy millipore filter was clamped between the Ussing Chambers. The apical and basolateral sides were filled with solutions to be used in the subsequent experiment (for instance, apical Cl^- free and basolateral Cl^- containing solution). The offset potential between the two 3M KCl agar bridges on each side of the filter (which includes the diffusion potential due to unilateral ion replacement), was compensated by adjusting the input offset of the amplifier. The fluid resistance associated with unilateral ion replacement was also compensated. After these procedures, the epididymal epithelium was clamped and bathed with solutions used in the correction of offset potential and fluid resistance. When the effects of transepithelial potential difference on Cl^- transport were investigated, the tissues were clamped intermittantly (1 every 20 seconds) from the short-circuit condition ($PD_t = 0$ mV) to various PD_t from +30 to -30 mV (apical side with reference to the basolateral side). The current across the epithelia (clamping current) was recorded on the chart-recorder. Inward current (the passage of anions from the basolateral to the apical side) was defined as positive and outward current (the passage of anions from the apical to the basolateral side) was defined as negative.

Tissue conductance was calculated from the clamping current and the potential difference applied (Chapter II.2.).

The I_{SC} response to adrenaline (ΔI_{SC}), expressed as $\mu eqhr^{-1}cm^{-2}$, was defined as the increase in area-under-curve integrated over 30 minutes after adrenaline stimulation. The clamping current response (ΔI_{CL}), expressed as μAcm^{-2} , was taken as the maximum increase in clamping current upon adrenaline stimulation. The conductance response to adrenaline (ΔG_t), expressed as $mScm^{-2}$, was the difference between the conductance measured at the peak of the clamping current response and that before stimulation. The area-under-curve were measured using an IBM compatible computer equipped with a digitizer (Hipad, Houston Instruments, Austin, Texas, U.S.A.) and the software Autocad®.

All the experiments were performed in HEPES-buffered Krebs solution in which 25 mM NaHCO3 was substituted by 25 mM NaHEPES. In the anion selectivity experiments, NaCl in the apical solution was progressively substituted by the Na salts of Br^-, I^-, NO_3^-, isethionate or gluconate. $CaCl_2$ and KCl were replaced with the respective salts of gluconate. In other experiments, gluconate was used as the sole Cl^- substituent. In all circumstances, the basolateral Cl^- concentration was unchanged (126.7 mM).

Results

Fig.III.5.1.i shows the effects of adrenaline on I_{SC} when both the apical and the basolateral solutions contained 126.7 mM of Cl^-. In ii) all apical Cl^- was substituted by gluconate. iii) shows the record when all apical Cl^- was replaced by gluconate but the tissue was pretreated apically with 1.0 mM of DPC. In all three situations, adrenaline gave rise to a transient rise in I_{SC} followed by a sustained phase which was higher than the

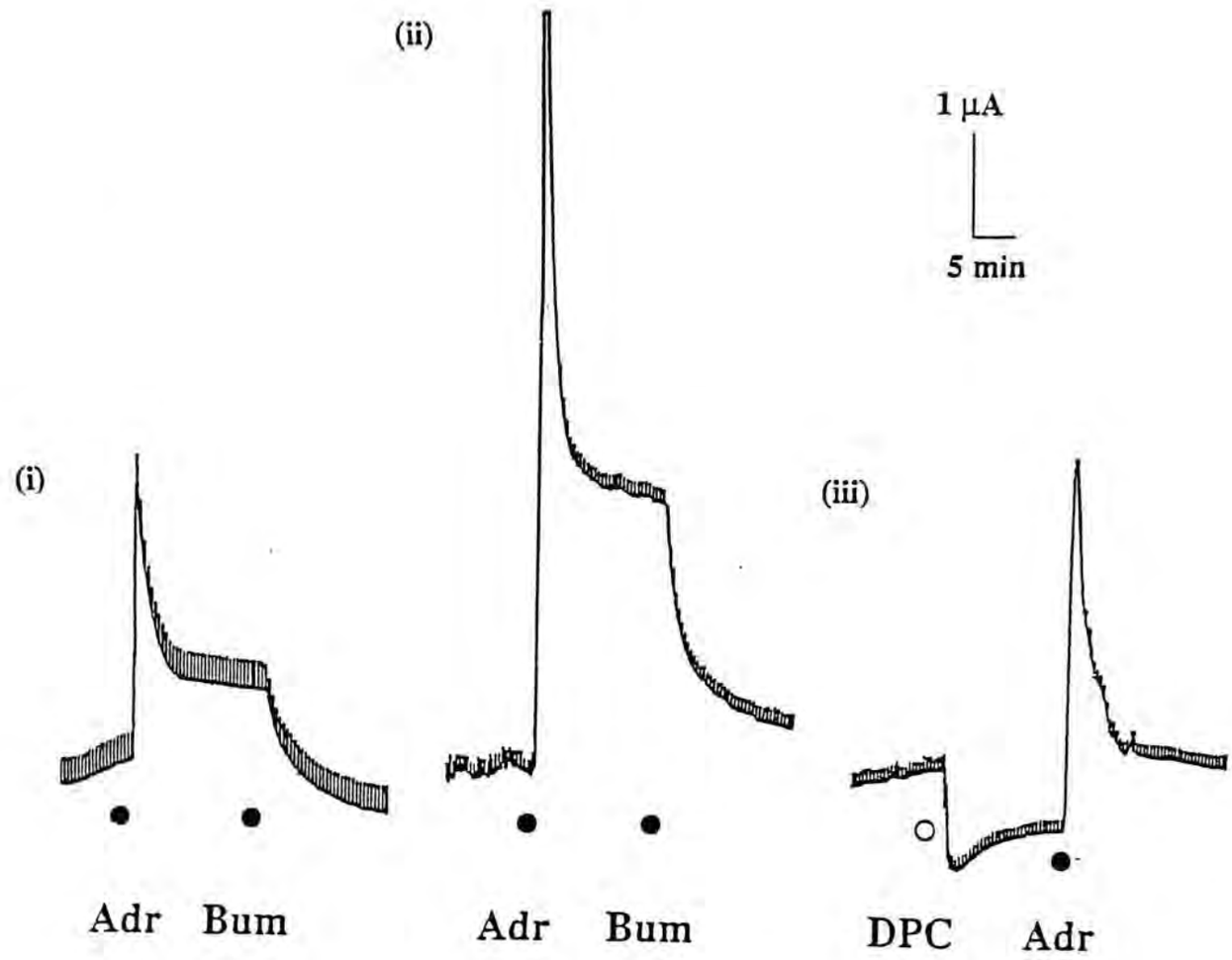

Fig.III.5.1

Short-circuit current measurement in three separate epididymal monolayers, area 0.4-0.5 cm^2. (i) Both the apical and basolateral sides of the tissue were bathed with HCO_3^--free, HEPES-buffered solution containing 126.7 mM of chloride. Adrenaline (Adr, 0.23 μM) was added to the basolateral bathing solution (closed circle) followed by bumetanide (Bum, 20 μM), also to the basolateral solution (closed circle). (ii) same as (i) except that the apical chloride concentration was reduced to zero by substitution with gluconate. (iii) The apical chloride concentration was reduced to zero by substitution with gluconate but the tissue was pretreated with 1 mM of DPC on the apical side (open circle) before adrenaline (0.23 μM) stimulation (closed circle). The current deflections superimposed on the I_{SC} record represented excursions of the voltage clamp (0.1-0.3 mV). The magnitude of deflection was related directly to tissue conductance. However, the size of deflection recorded on chart paper was too small for an effect of adrenaline on tissue conductance to be discerned. Each record is representative of 4 different experiments.

prestimulated level. Bumetanide (20 μM) added basolaterally almost abolished the adrenaline stimulated I_{SC}.

Fig.III.5.2. shows the average ΔI_{SC} measured at various $[Cl^-]_a$ when the latter was progressively reduced from 126.7 to 0 mM by substituting apical Cl^- with gluconate. Decreasing $[Cl^-]_a$ increased the I_{SC} response to adrenaline in a linear fashion with a slope of -1.6×10^{-3} $\mu eqhr^{-1}cm^{-2}$ per mM change in $[Cl^-]_a$ (coefficient of determination $r^2 = 0.97$). Pretreatment with DPC significantly ($p < 0.05$) reduced the slope to -4.9×10^{-4} $\mu eqhr^{-1}cm^{-2}$ per mM change in $[Cl^-]_a$ ($r^2 = 0.877$).

Fig.III.5.3. shows the average ΔI_{SC} measured at various $[Cl^-]_a$ when apical Cl^- was progressively substituted with isethionate, gluconate, I^-, Br^- as well as NO_3^-. With isethionate as apical Cl^- substituent, ΔI_{SC} increased with decreasing $[Cl^-]_a$ in a curvilinear fashion and the curve intersected the y-axis at 0.410 ± 0.02 $\mu eqhr^{-1}cm^{-2}$. When gluconate or I^- was used, ΔI_{SC} increased with decreasing $[Cl^-]_a$ in a linear fashion with slopes of -1.6×10^{-3} and -0.2×10^{-3} $\mu eqhr^{-1}cm^{-2}$ per mM change in $[Cl^-]_a$ and y-intercepts of 0.23 ± 0.03 and 0.08 ± 0.001 $\mu eqhr^{-1}cm^{-2}$ respectively. On the other hand, when Br^- or NO_3^- was used, ΔI_{SC} increased with increasing $[Cl^-]_a$ in a linear fashion with slopes of 1.2×10^{-4} and 1.8×10^{-4} $\mu eqhr^{-1}cm^{-2}$ per mM change in $[Cl^-]_a$ and y-intercepts of 0.043 ± 0.005 and 0.043 ± 0.03 $\mu eqhr^{-1}cm^{-2}$ respectively. To compare the relative selectivity among anions, the ΔI_{SC} measured at $[Cl^-]_a = 126.7$ mM (i.e. no Cl^- substituent, $\Delta I_{SC} = 0.066 \pm 0.002$ $\mu eqhr^{-1}cm^{-2}$) was divided by the y-intercepts of the curves ($[Cl^-]_a = 0$ mM) shown in fig.III.1.3.. The permeability sequence was found to be NO_3^- (1.5) ~ Br^- (1.5) > Cl^- (1.0) > I^- (0.8) > Gluconate (0.3) > Isethionate (0.2). For statistical evaluation, the changes in I_{SC} when various substituent anions completely replaced apical Cl^- (i.e. the y-intercepts) were compared using Student's unpaired t-test. When comparisons between

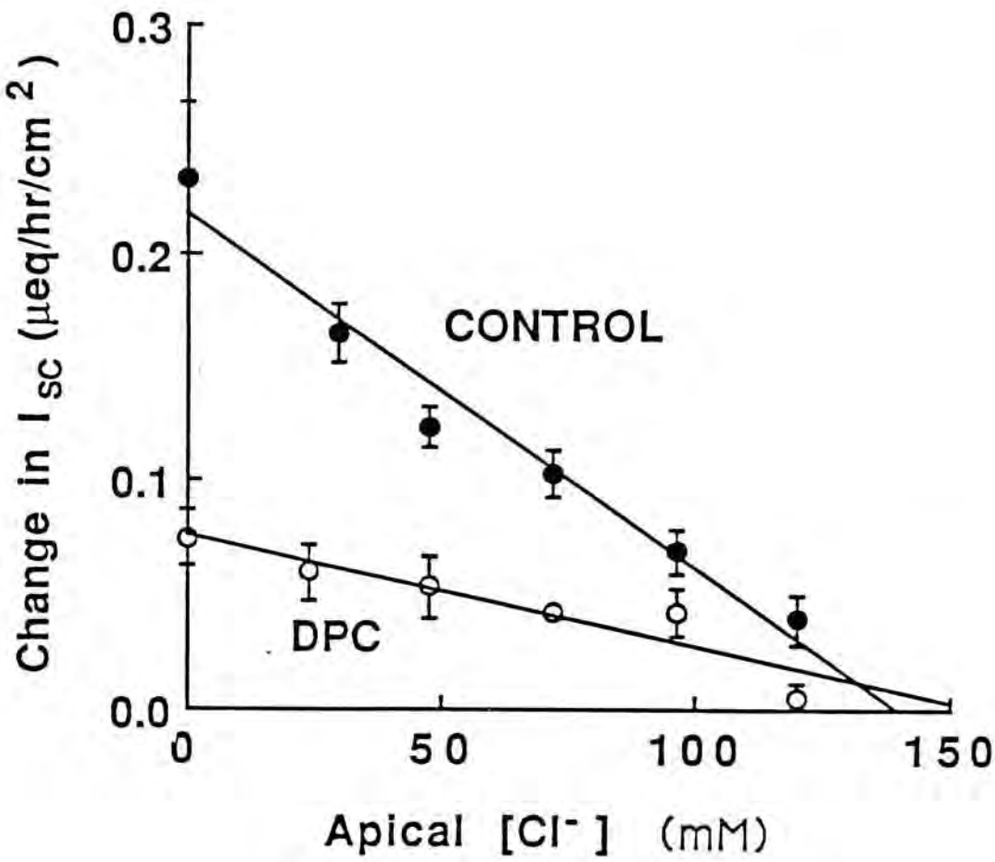

Fig.III.5.2.

The short-circuit current response to adrenaline at different apical chloride concentrations when the tissues were bathed with HCO_3^--free, HEPES-buffered solution. Adrenaline (0.23 μM) was added to the basolateral side with (open circles) and without (closed circles) DPC (1 mM) pretreatment. Values shown are the S.E. of mean for 4 to 6 cultures.

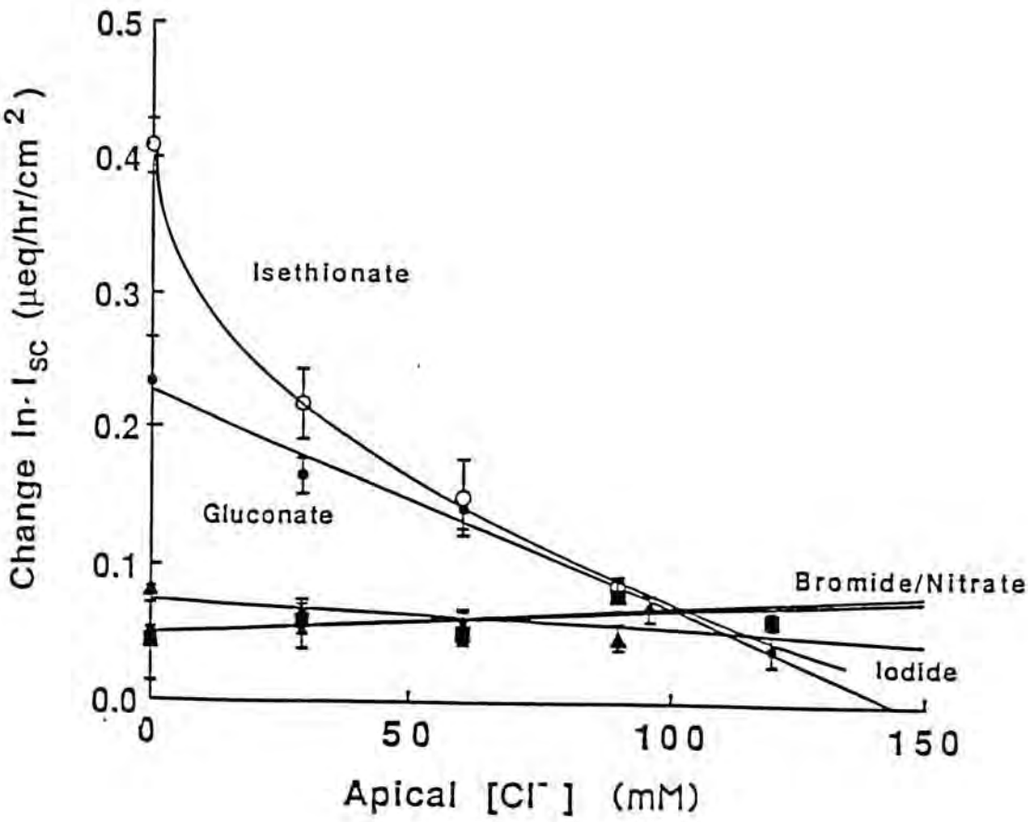

Fig.III.5.3.

The short-circuit response to adrenaline at different apical chloride concentrations when chloride was substituted by isethionate (open circles), gluconate (closed circles), iodide (closed triangles), bromide (squares) and nitrate (open triangles) in HCO_3^--free, HEPES-buffered solution . Adrenaline (0.23 μM) was added to the basolateral side of the tissue. Values shown are the S.E. of mean for 4 to 6 cultures.

the substituent anions and Cl^- were made, the y-intercepts were compared with the ΔI_{SC} when apical Cl^- was not substituted at all (i.e. $\Delta I_{SC} = 0.066 \pm 0.002$ $\mu eqhr^{-1}cm^{-2}$). It was found that the differences between Br^- and Cl^-, Cl^- and I^-, I^- and gluconate as well as gluconate and isethionate, were statistically significant ($P < 0.05$ in each case). However, the differences between NO_3^- and Br^- and between NO_3^- and Cl^- were not statistically significant.

Studies were also carried out to investigate the voltage-dependence of transepithelial Cl^- secretion. Fig.III.5.4.i. shows the effects of adrenaline on the clamping current when the tissue was bathed on both sides with 126.7 mM Cl^- and was clamped intermittantly at +30 mV (lumen positive) and 0 mV. At +30 mV, adrenaline gave rise to an increase in current which approached a maximum within 1 minute after stimulation. The clamping current then levelled off to a plateau value higher than the prestimulated level. The current response to adrenaline under short-circuit condition ($PD_t = 0$ mV) was comparatively smaller. ii) shows the record of a similar experiment in which 2 mM of DPC was added on the apical side before adrenaline stimulation. DPC gave rise to a transient fall in clamping current which then increased to a level slightly higher than the prestimulated current. The current response to subsequent stimulation with adrenaline was much reduced as compared to (i). At 0 mV, the current response to adrenaline was completely abolished. Fig.III.5.5.i. shows the effects of adrenaline when the tissue was clamped intermittantly at - 30 mV (lumen negative) and 0 mV. Addition of adrenaline gave rise to a negative current response at -30 mV. At 0 mV, adrenaline gave rise to a small increase in current . ii) shows the record of a similar experiment in which the tissue was pretreated with 2 mM of DPC on the apical side before the adrenaline stimulation. DPC gave rise to a transient fall in clamping current (less negative) which then increased to a level more negative than the prestimulated level. DPC pretreatment reduced the current response to adrenaline as compared to (i). At 0 mV, the current response to adrenaline was completely

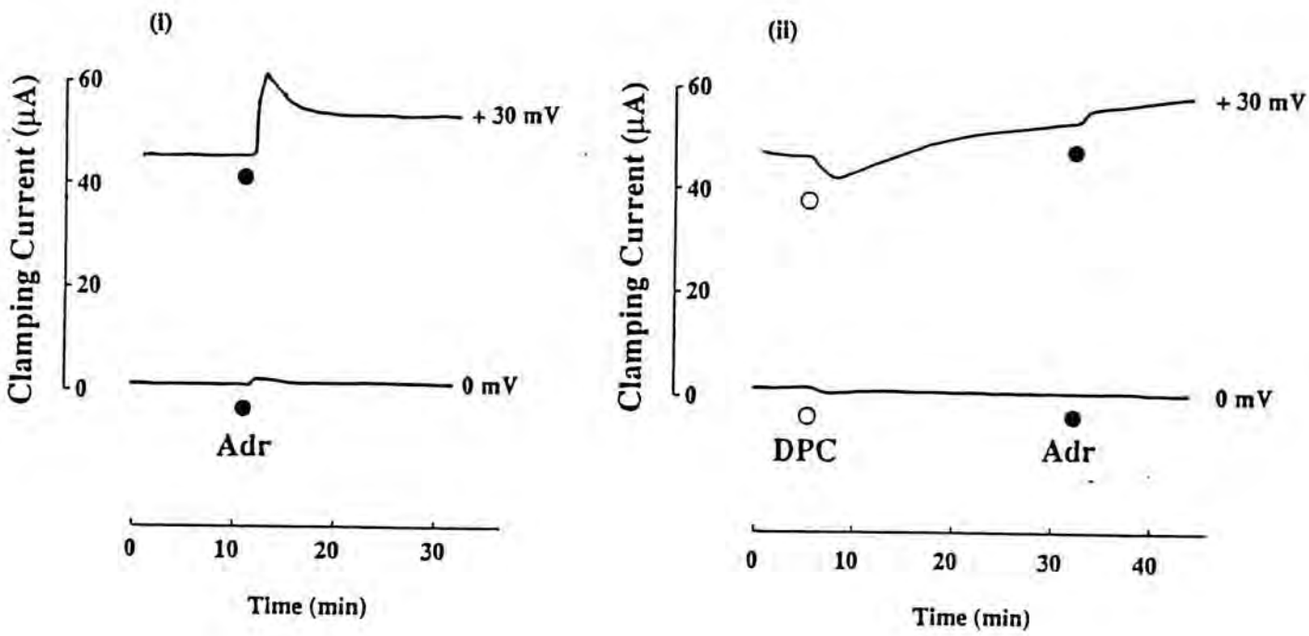

Fig.III.5.4.

Clamping current (I_{CL}) measured in two separate monolayers, area 0.4-0.5 cm^2, when the tissues were clamped between 0 mV and +30 mV (lumen positive) in HCO_3^--free, HEPES-buffered solution . (A) Adrenaline (Adr, 0.23 μM) added to the basolateral side (closed circles). (B) same as (A) except that the tissue has been pretreated with DPC (DPC, 2 mM) on the apical side (open circles) before adrenaline stimulation. Note that the scale of the current is different from those of the other figures. Each record is representative of 4 different experiments.

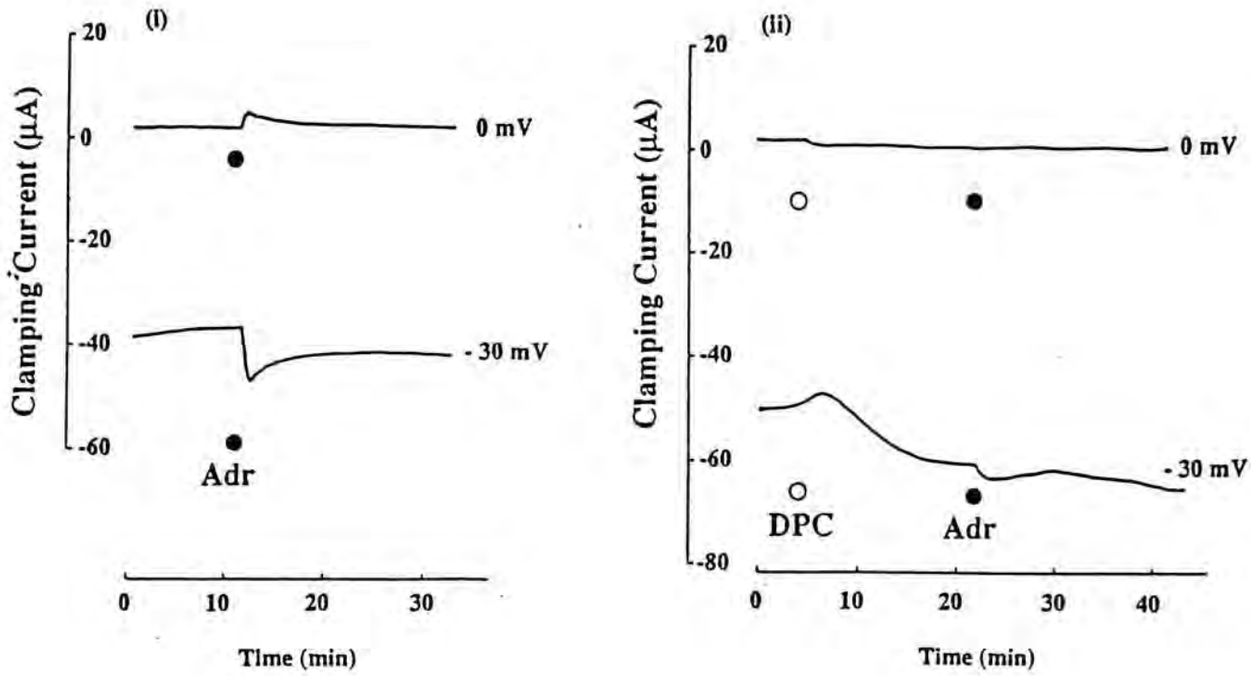

Fig.III.5.5.

Clamping current (I_{CL}) measured in two separate monolayers, area 0.4-0.5 cm^2, when the tissues were clamped between 0 mV and -30 mV (lumen negative) in HCO_3^--free, HEPES-buffered solution . (i) Adrenaline (Adr, 0.23 μM) added to the basolateral side (closed circles). (ii) same as (i) except that the tissue has been pretreated with DPC (DPC, 2 mM) on the apical side (open circles) before adrenaline stimulation. Each record is representative of 4 different experiments.

abolished. Fig.III.5.6.i. shows the effects of adrenaline when the tissues were bathed on both sides with 126.7 mM Cl^- and were clamped at different PD_t (apical with respect to basolateral). The prestimulated clamping currents at various PD_t were deliberately aligned at the same level so that the current responses to adrenaline could be compared. Changing the PD_t from +30 (lumen positive) to 0 mV reduced ΔI_{CL} in a voltage dependent fashion. At more hyperpolarized PD_t from -5 to -30 mV (lumen negative), adrenaline gave rise to decreases in I_{CL} (i.e. negative ΔI_{CL}). ii) shows the records at $[Cl^-]_a$ = 0 mM. Changing the PD_t from +30 mV (lumen positive) to - 30 mV (lumen positive) decreased ΔI_{CL}. However, ΔI_{CL} was positive at all PD_t tested and the dependence of ΔI_{CL} on PD_t was less than that in (i).

Fig.III.5.7. shows the average ΔI_{CL} at various PD_t. When the tissues were bathed on both sides with 126.7 mM Cl^-, ΔI_{CL} changed with PD_t in a linear fashion. Positive ΔI_{SC} was observed at lumen postive PD_t and negative ΔI_{SC} was observed at lumen negative PD_t. Zero ΔI_{SC} was obtained at PD_t = -6 mV. When $[Cl^-]_a$ was reduced to 0 mM, the dependence of ΔI_{CL} on PD_t was less and ΔI_{CL} was positive at all PD_t tested. Fig. III.5.8 shows the average transepithelial conductance response to adrenaline (ΔG_t) at various PD_t. It can be seen that ΔG_t did not vary with the PD_t applied. Fig.III.5.9. shows the average ΔG_t at various apical chloride concentrations. Results at various PD_t have been pooled together. ΔG_t decreased as $[Cl^-]_a$ was reduced with a correlation coefficient of 0.81 ($p < 0.05$).

Discussion

In the present study, the I_{SC} response to adrenaline (ΔI_{SC}) was taken as an indication of transepithelial Cl^- transport. Since Cl^- was actively accumulated inside the cells at the basolateral membrane by the electroneutral $Na^+/K^+/2Cl^-$ cotransport and/or the HCO_3^-/Cl^- exchange, ΔI_{SC} could be attributed to the electrogenic diffusion of Cl^- from the

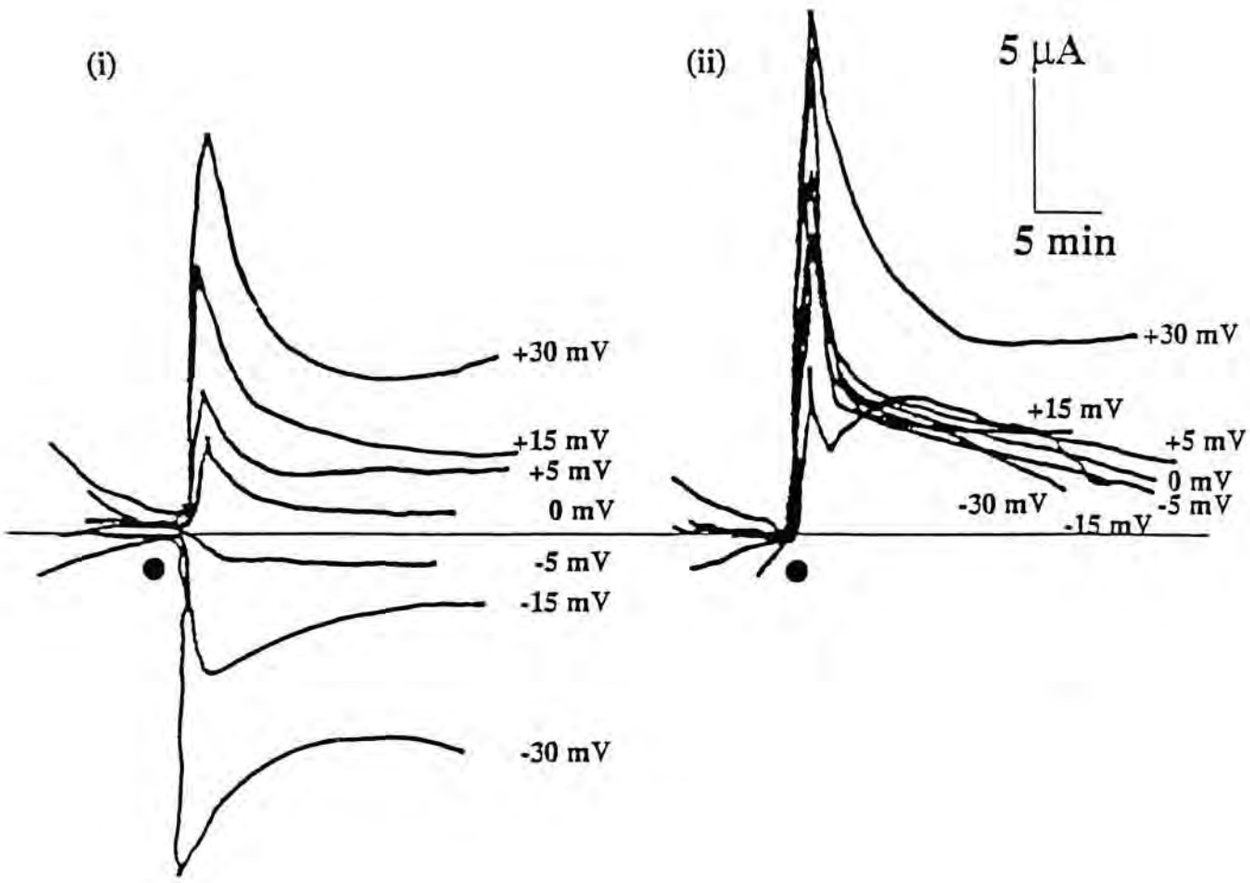

Fig.III.5.6.

Clamping current response to adrenaline (0.23 μM, closed circles) measured in fourteen separate monolayers, area 0.4-0.5 cm^2, when individual monolayer was clamped at a particular transepithelial potential difference between +30 mV and -30 mV (luminal with respect to the basolateral side) in HCO_3^--free, HEPES-buffered solution. The clamping current levels before adrenaline stimulation in different tissues were deliberately aligned at the same level (the horizontal line at the middle of the figure) so that the current response to adrenaline could be compared. (i) The apical and the basolateral bathing solutions contained 126.7 mM chloride. (ii) The apical chloride concentration was reduced to zero by substituting chloride with gluconate. Each record is representative of 4 different experiments.

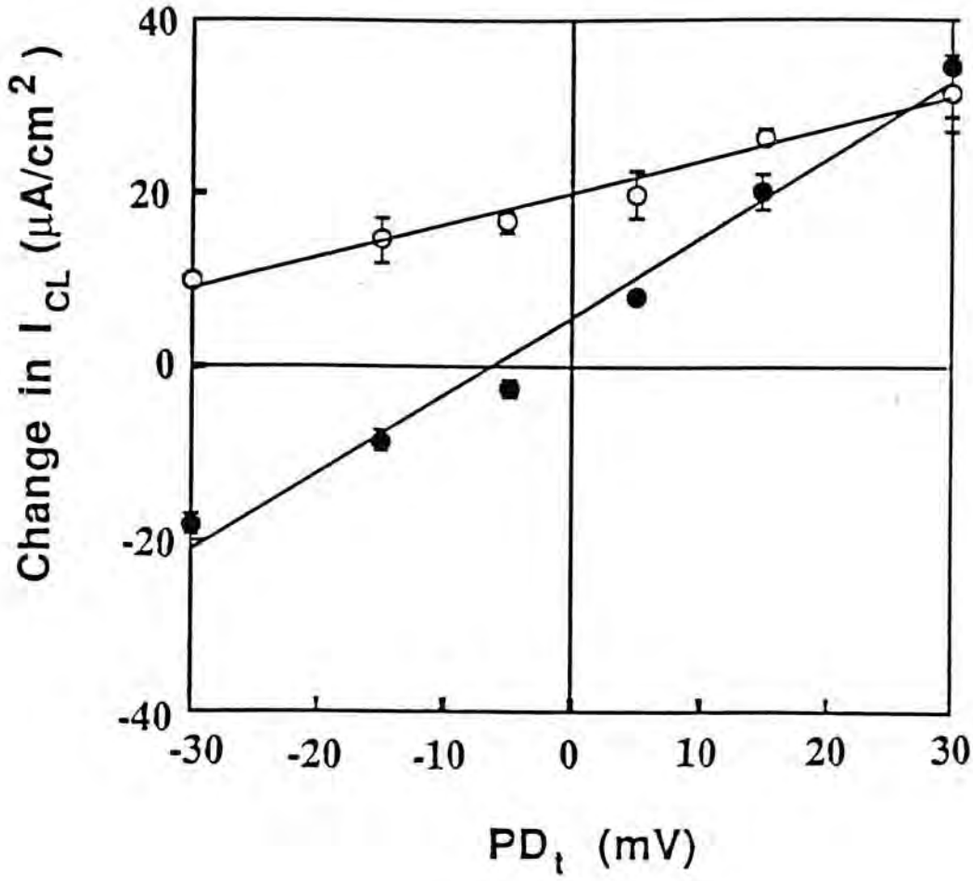

Fig.III.5.7

Effect of varying the transepithelial potential difference (PD_t) on the clamping current response to adrenaline in HCO_3^--free, HEPES-buffered solution when the apical side contained 126.7 mM chloride (closed circles) or 0 mM chloride (open circles) by substituting chloride with gluconate. Values shown are S.E. of mean for 4 to 6 cultures.

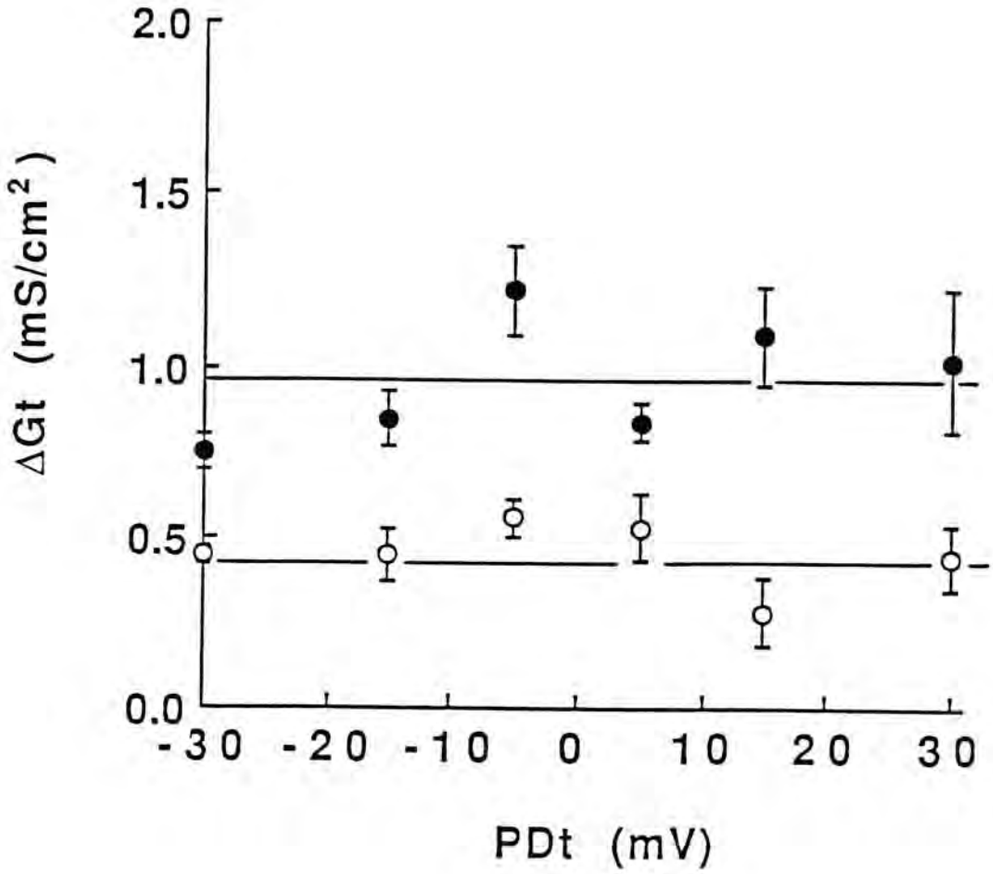

Fig.III.5.8

Effect of varying the transepithelial potential difference (PD_t) on the transepithelial conductance (ΔG_t) response to adrenaline in HCO_3^--free, HEPES-buffered solution when the apical side contained 126.7 mM chloride (closed circles) or 0 mM chloride (open circiles). Values shown are S.E. of mean for 4 to 6 cultures.

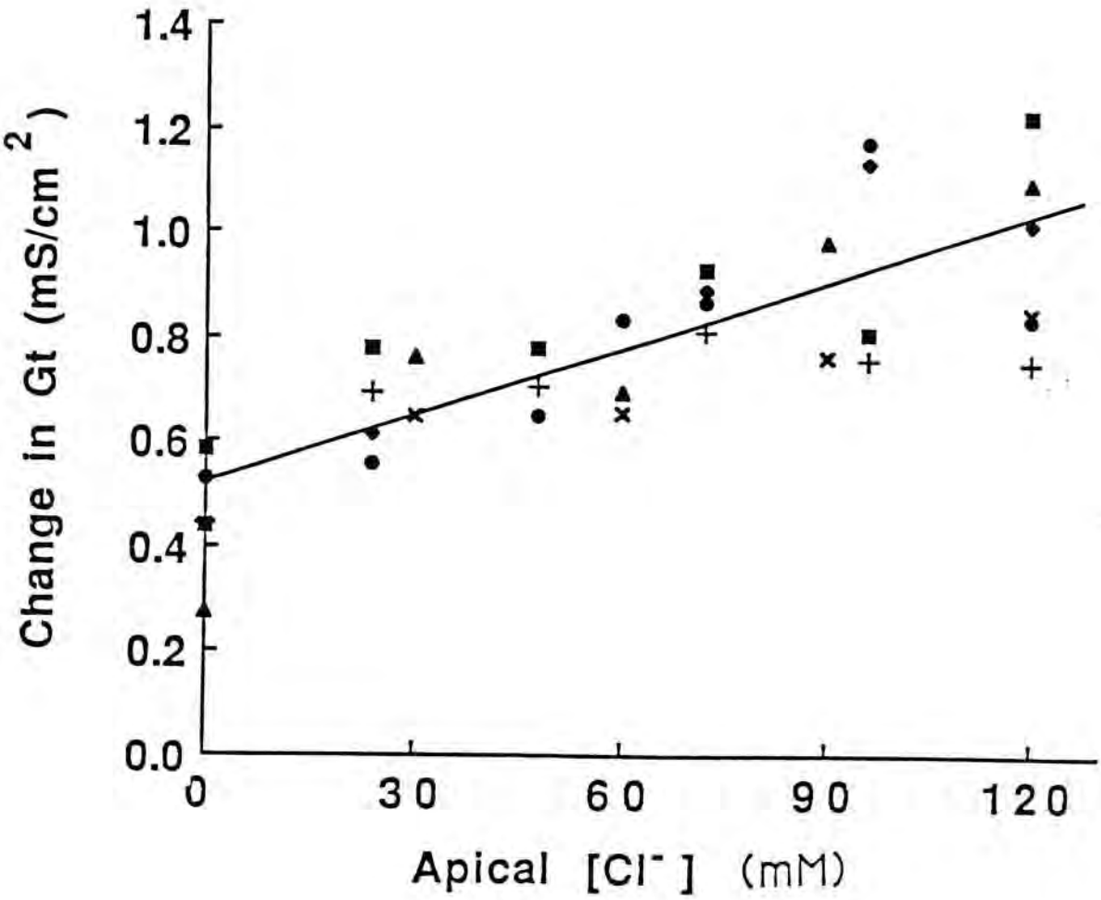

Fig.III.5.9

The transepithelial conductance response (ΔG_t) to adrenaline at different apical chloride concentrations (substituted by gluconate). The results at various transepithelial potential differences were pooled and were indicated by different symbols in the figure (•, +5 mV; , -5 mV; Δ, +15 mV; x, -15 mV; ,+30 mV; +, -30 mV, apical with reference to basolateral side). Each point in the figure is the mean of 4 to 6 cultures.

cytosol into the apical solution via apical Cl^- channels (Wong, 1988a&c). This was supported by the fact that apical application of a Cl^- channel blocker DPC greatly diminished the I_{SC} response to adrenaline (Fig.III.5.2). Moreover, bilateral removal of Cl^- has been shown to reduce the effect of adrenaline (Wong, 1988a), which was expected if Cl^- was the major ion species responsible for the I_{SC}. On the other hand, Na^+ absorption did not seem to play a significant role in I_{SC} as the apical application of the Na^+ channel blocker amiloride at a concentration of 0.1 mM did not affect the basal I_{SC} or the I_{SC} response to adrenaline (Wong, 1988a). Circumstantial evidence has shown that adrenaline increased Cl^- permeability of the apical membrane (Klyce & Wong, 1977; Welsh et al., 1983) without significant effect on the paracellular permeability (Nagel & Reinach 1980; Pederson, 1990) or the transepithelial Na^+ transport (Al-Bazzaz & Cheng, 1979).

Decreasing apical Cl^- concentration was found to increase the I_{SC} response to adrenaline. The results were in keeping with those of Cotton et al. (1988); Shorofsky et al. (1983); Simmons (1991) and Willumsen & Boucher (1989). To avoid the complication due to transepithelial diffusion potential as a result of the concentration gradient across the epithelium, only the increase in current upon stimulation with adrenaline was considered. The enhanced response at low $[Cl^-]_a$ might be attributed to increased Cl^- gradient across the apical membrane. This is supported by the fact that apical application of DPC reduced the enhanced response to adrenaline. Furthermore, bumetanide, which inhibits Cl^- uptake through the basolateral $Na^+/K^+/2Cl^-$ symport (Wong, 1988a), was found to reduce the adrenaline stimulated I_{SC} response at $[Cl^-]_a = 0$ mM (see Fig.III.5.1.).

Anion channels in different tissues have been shown to exhibit selectivity towards various anions. Most studies on anion selectivity have been restricted to the single-cell and/or single-channel level. Studies on intact epithelium have not been satisfactory because the transepithelial anion secretion is limited by the selectivity of the basolateral $Na^+/K^+/2Cl^-$

symport (Widdicombe & Welsh, 1980). It is proposed that when apical Cl^- concentration was decreased by substituting Cl^- with an equimolar amount of impermeant anions, the driving force for Cl^- exit across the apical membrane increased. Upon stimulation with adrenaline, the increase in inward current, i.e. ΔI_{SC} (carried by the Cl^- efflux from the cytosol to the apical solution) would be enhanced. If the substituent was as permeant as Cl^-, the enhanced ΔI_{SC} would be counterbalanced by the influx of permeant substituent occurred in a direction opposite to the Cl^- efflux. The more the apical Cl^- was substituted, the greater the driving force for Cl^- exit across the apical anion conductance yet at the same time, a more favourable concentration gradient for the influx of the substituent from the apical solution into the cytosol was created. Therefore, ΔI_{SC} should be independent of apical Cl^- concentration. On the other hand, if the substituent was more permeant than Cl^-, the influx of the permeant substituent would overcome the enhanced ΔI_{SC} due to Cl^- efflux and the overall ΔI_{SC} would be less than if apical Cl^- was not substituted at all. The present study showed that the selectivity sequence of apical anion conductance upon stimulation with adrenaline stimulation in the epididymal epithelium was $NO_3^- \sim Br^- > Cl^- > I^-$ > gluconate > isethionate. Using the whole-cell patch-clamp technique, similar selectivity sequence has been found in the cAMP-mediated Cl^- conductance in the epididymal (Huang et al.,1992a) and colonic tumour cells (Cliff & Frizzell, 1990), the genetic defect of which is thought to be the cause of cystic fibrosis (Chapter I.5.).

The present study investigated the voltage-dependence of Cl^- transport across the epithelium. It was found that the clamping current response to adrenaline (ΔI_{CL}) changed with the PD_t applied in a linear fashion. When the tissue was stimulated with adrenaline at lumen positive PDt, chloride is driven from the cytosol into the apical solution along the electrochemical gradient. This gave rise to an increase in I_{CL}. At lumen negative PDt, Cl^- transport became opposite in direction, that is, from the apical solution back to the cytosol and I_{CL} decreased upon stimulation with adrenaline. It is worth noting that at $PD_t = 0$

mV, adrenaline gave rise to positive ΔI_{SC} and zero ΔI_{CL} was observed at PD_t = -6 mV. If adrenaline exerted its effect nonspecifically on the paracellular pathway, zero ΔI_{CL} would be expected at PD_t = 0 mV at which the driving force for passive Cl^- transport across the epithelium became zero. A positive ΔI_{SC} at zero PD_t indicated that adrenaline stimulated trancellular Cl^- transport instead of increasing the paracellular permeability. Both Cl^- efflux and influx involved apical Cl^- channels as both processes were blocked by a Cl^- channel blocker DPC, in the apical solution (Fig.III.5.5. & III.5.6.). This contention was further supported by the evidence that reducing apical Cl^- to zero reduced the dependence of ΔI_{CL} on PD_t such that ΔI_{CL} was positive (efflux) at all PD_t tested (Fig.III.5.7. & III.5.8) as was expected as there was virtually no Cl^- for influx to take place.

Another finding in this study was the dependence of the transepithelial conductance response to adrenaline (ΔG_t) on apical Cl^- concentration. It was found that ΔG_t did not vary with the PD_t applied but was reduced with decreasing apical Cl^- concentration. The results were in contrast to those in frog skin which showed considerable increase in Cl^- conductance at mucosal (luminal) negative PD_t (Larsen & Kristensen, 1978) but were in keeping with those of Welsh (1985) who found that the increase in apical conductances upon adrenaline stimulation in tracheal epithelium were insensitive to the transepithelial voltages applied. Recent studies using the whole-cell patch-clamp technique have also shown that the cAMP-mediated Cl^- conductance in rat epididymal cells was voltage-insensitive (Huang et al., 1992a). That the apical Cl^- conductance was reduced by apical Cl^- removal has been observed in reabsorptive sweat duct epithelium (Reddy & Quinton, 1989), frog skin (Larsen et al., 1987; De Wolf et al., 1989) as well as the rabbit corneal epithelium (Klyce & Wong, 1977). It was speculated that decrease in $[Cl^-]_a$ led to fall in intracellular Cl^- concentration. The latter might give rise to feedback inhibition to reduce the apical Cl^- conductance in order to prevent further Cl^- secretion.

Chapter III.6

Effects of secretory agonists on transepithelial Cl^- transport and intracellular Ca^{2+} concentration in cultured human epididymal epithelium

Introduction

Studies on electrolyte transport by the rat cauda epididymidis have been facilitated by the ability to grow the cells on pervious supports (Chapter II.1). As the epididymis is responsible for the development of sperm fertility (Cooper, 1986), understanding human epididymal functions is important for the treatment of idiopathic male infertility and the development of post-testicular contraceptive agents (Chapter I.6). The present study investigated the effects of secretory agonists on transepithelial Cl^- secretion by cultured human epididymal epithelium and the effects of Ca^{2+} mobilizing agonists on $[Ca^{2+}]_i$ in single human epididymal cells.

Methods

Tissue culture technique

Human epididymides were obtained from patients suffering from prostatic carcinoma who underwent orchidectomy as part of the treatment. Orchidectomy was operated by the Urology Section of the Department of Surgery in the Prince of Wales Hospital in Hong Kong. The tissue was ice-cooled and brought from the hospital to the laboratory within 30 minutes. The procedures of cell culture were modified from those described by Cooper et al. (1990). Upon arrival, the tissue was immersed in HBSS in a 10 cm Petri dish. The adipose tissue as well as the epididymal capsule were removed with fine forceps under a dissecting microscope (Model SZM, Kyowa, Japan). The mid portion (corpus plus a part of the cauda) of the epididymis was dissected out and transferred to a culture flask containing 0.8 % (w/v) collagenase and 0.005 % (w/v) elastase in 10 ml

HBSS and was shaken at 160 strokes/min at 32 ^{o}C for 15 minutes. The contents of the flask were transferred into a 10 cm Petri dish and the peritubular connective tissue was torn away with fine forceps. The epididymal tissue was transferred back to the culture flask and the alternating processes of connective tissue tearing and enzyme digestion were repeated twice. Afterwards, the processes were repeated three times with 10 ml HBSS containing fresh collagenase and elastase and the epididymal tubules were then finely chopped with scissors and were transferred to a culture flask containing 0.8 % collagenase and 0.1 % hyaluronidase in 10 ml HBSS. The contents were shaken at 160 strokes/min at 32^{o}C for 45 minutes and centrifuged at 800 g for 5 minutes. The supernatant was removed by suction and the pellet of cells was resuspended in 10 ml HBSS containing fresh collagenase and hyaluronidase. The process of enzyme digestion continued for another 45 minutes. The tissue was washed in 10 ml EMEM for several times until all the collagenase and hyaluronidase in the EMEM was removed. Finally, the tissue was resuspended in 8 ml EMEM. 0.25 ml of the cell suspension was plated onto each pervious support floating on a 35 mm Petri dish containing 2 ml EMEM. For microfluorimetric measurement, the cell suspension was plated onto glass coverslips. The cultures were incubated undisturbed at 32^{o}C for 10 days. Thereafter, epididymal cells grown on coverslips were ready for Ca^{2+} measurement. For short-circuit current measurement, the epididymal monolayers reached confluency after 50 days of culture and were ready for the measurement of the short-circuit current. Studies on human epididymal tissues have been approved by the clinical research ethical committee.

Results

In normal Krebs-Henseleit (K-H) Solution, cultured human epididymal epithelia exhibited an average transepithelial potential difference of 5.5 ± 1.3 mV (apical side negative) (n=16 cultures) and a basal I_{SC} of 6.9 ± 0.9 $\mu A/cm^2$ (n=16 cultures). Fig.III.6.1.i shows the effects of adrenaline on I_{SC} in normal K-H solution. Adrenaline

added basolaterally elicited a rise in I_{SC} consisting of a fast transient peak followed by a plateau level considerably higher than the basal level. Addition of a Cl^- channel blocker diphenylamine-2-carboxylate (DPC) on the apical side inhibited the adrenaline-induced I_{SC} response to the basal level. ii) shows the effect of adrenaline in Cl^--free solution in which Cl^- was substituted by gluconate. Removing ambient Cl^- reduced the peak I_{SC} response to adrenaline and the second phase of response became slowly declining.

It is widely believed that adrenaline elicits cellular response by acting on ß-adrenergic receptors, leading to a rise in intracellular cAMP by coupling to a membrane bound enzyme adenylate cyclase (Levitzki, 1988). Fig.III.6.2 shows the effects of forskolin, an activator of adenylate cyclase, on I_{SC}. Like adrenaline, forskolin elicited a rise in I_{SC} consisting of a rapid transient peak followed by a slowly increasing phase which attained a plateau level 45 minutes after stimulation. Subsequent addition of DPC led to a fall in I_{SC} to about the prestimulated level.

Increase in intracellular Ca^{2+} has been shown to stimulate transepithelial Cl^- secretion in rat epididymal epithelium (Wong, 1988a). Fig.III.6.3. shows the effects of a Ca^{2+} ionophore A23187 on I_{SC}. Addition of A23187 on the basolateral side had no effect while apical addition elicited a rapid transient rise in I_{SC} followed by a slowly declining phase. Addition of a $Na^+/ K^+/ 2Cl^-$ symport inhibitor bumetanide on the basolateral side gave rise to a further decrease in I_{SC}. Cl^- secretion in cultured rat epididymal epithelium has been shown to be stimulated by local factors like the calcitonin gene-related peptide (Chapter III.2.). Fig.III.6.4. shows the effects of CGRP on Cl^- secretion in cultured human epididymal epithelium. CGRP added basolaterally led to a rise in I_{SC} comprising of a rapid transient peak followed by a plateau level higher than the basal I_{SC}. Inhibition by DPC on the apical side led to a fall in I_{SC} below the prestimulated level.

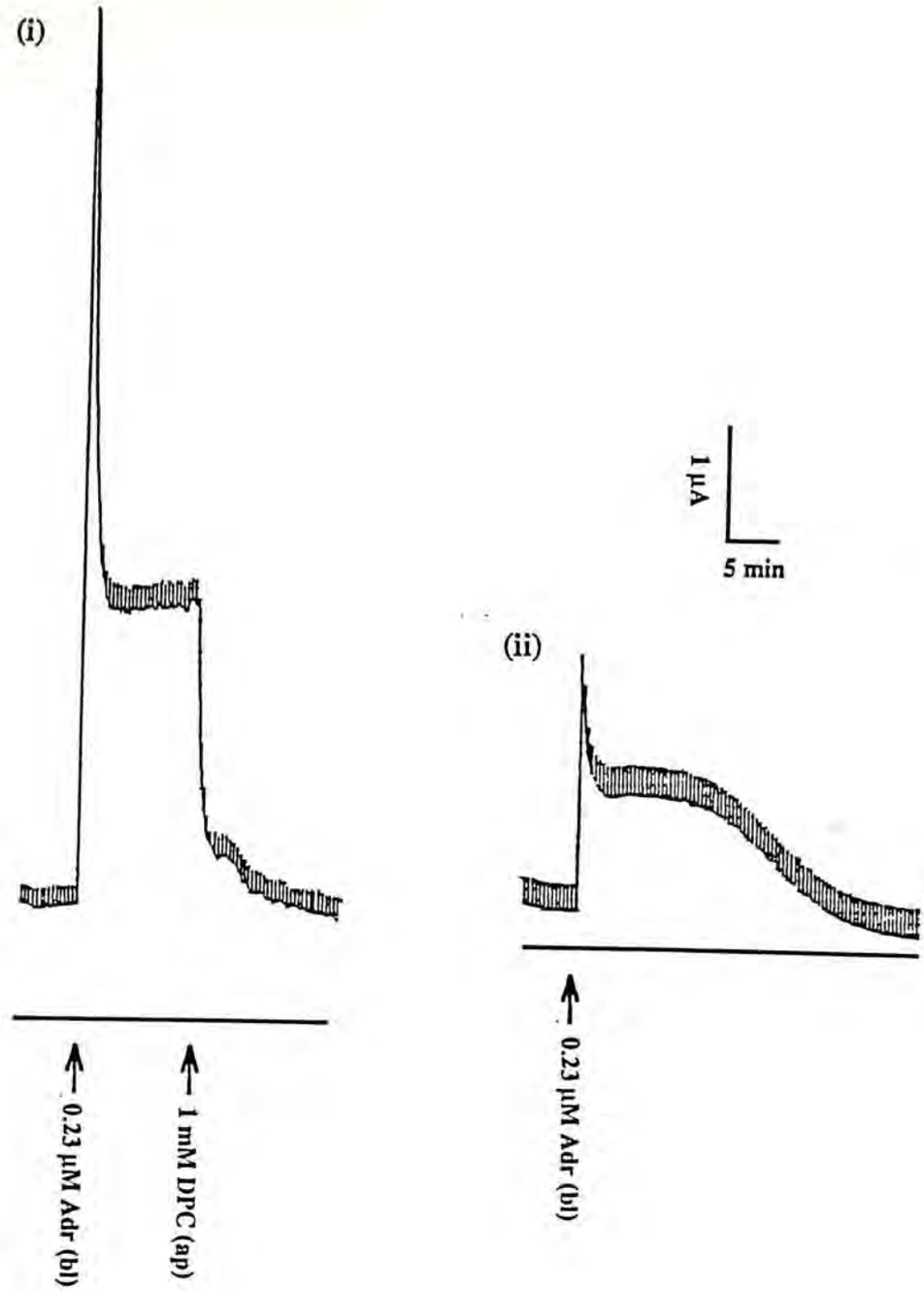

Fig.III.6.1.

Effects of adrenaline on I_{SC} in cultured human epididymal epithelium. (i) In normal K-H solution, adrenaline (0.23 μM) added to the basolateral side caused a rise in I_{SC} consisting of a rapid transient phase followed by a sustained response. The I_{SC} response could be inhibited by the apical application of a Cl^- channel blocker, DPC (1 mM). (ii) In Cl^--free solution, the respone to adrenaline was much reduced. The arrows indicate the time when adrenaline and DPC were added.

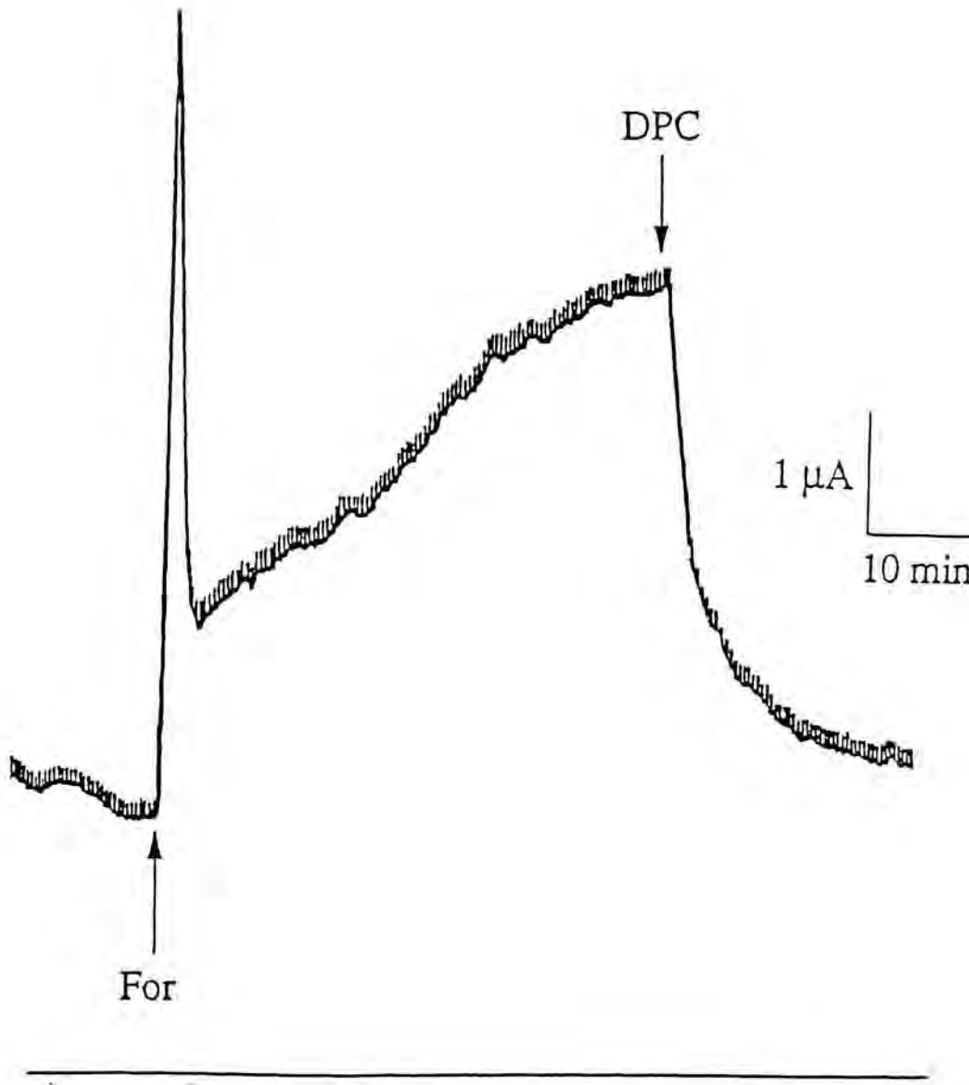

Fig.III.6.2.

Effects of forskolin on I_{SC} in cultured human epididymal epithelium. Forskolin (1 μM) added to the basolateral side caused a rise in I_{SC} consisting of a rapid transient phase followed by a slowly rising response. The I_{SC} response could be inhibited by the apical application of a Cl^- channel blocker, DPC (1 mM). The arrows indicate the time when forskolin and DPC were added.

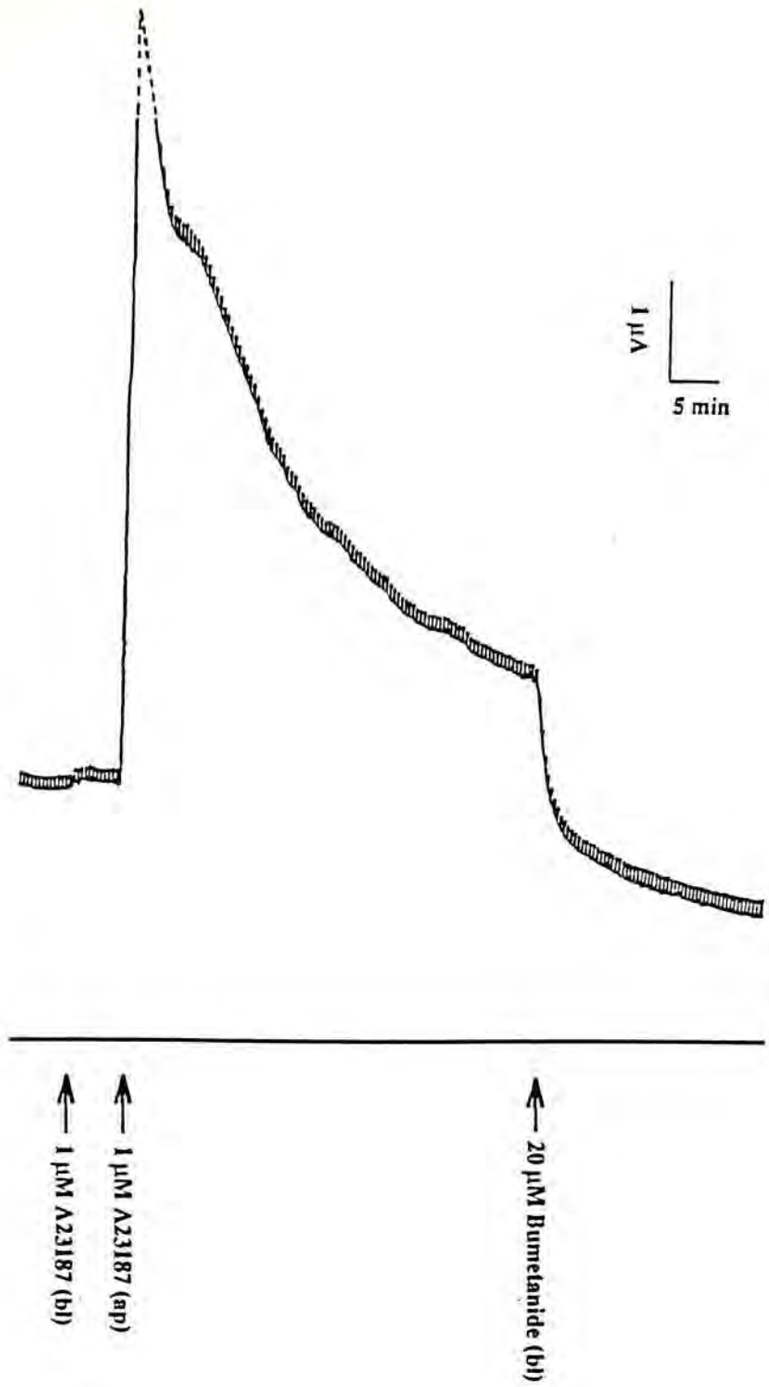

Fig.III.6.3

Effects of a calcium ionophore, A23187, on I_{SC} in cultured human epididymal epithelium. A23187 (1 μM) added to the apical side caused a rise in I_{SC} consisting of a rapid transient phase followed by a slowly decreasing response. The I_{SC} response could be inhibited by the basolateral application of a $Na^+/K^+/2Cl^-$ symport inibitor, bumetanide (20 μM). The arrows indicate the time when A23187 and bumetanide were added.

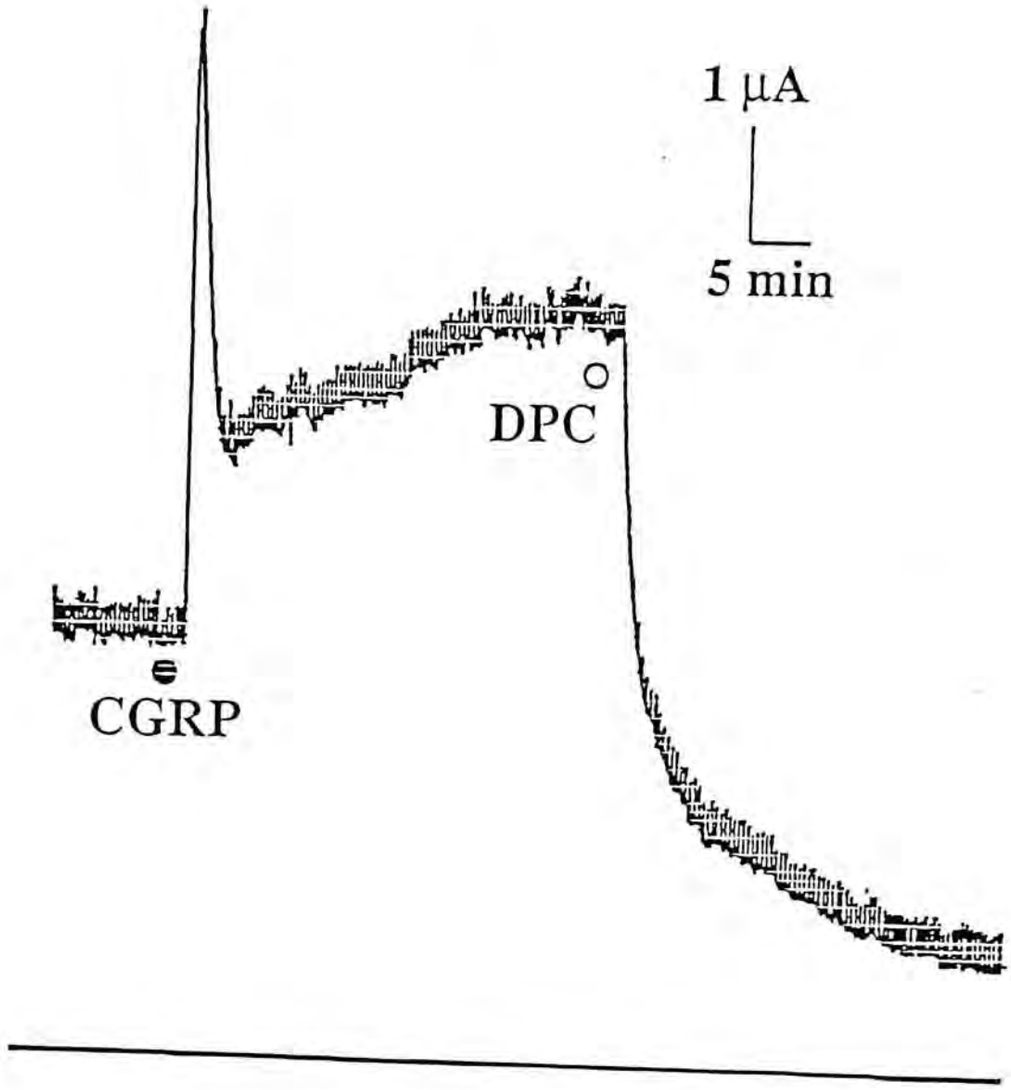

Fig.III.6.4

Effects of CGPR (1 μM) on I_{SC} in cultured human epididymal epithelium. The peptide was added to the basolateral aspect of the tissue. The I_{SC} response consisted of a rapid transient rise followed by a sustained response which could be inhibited by the apical application of Cl^- channel blocker, DPC (1 mM).

In rat epididymal cells, ATP has been shown to release Ca^{2+} from intracellular store, causing a rise in $[Ca^{2+}]_i$ (Chapter III.3). Microfluorimetric studies were carried out on single human epididymal cells to see if similar Ca^{2+} store exists in human tissue. Fig. III.6.5 shows that in normal HEPES-buffered solution, ATP (20 μM) elicited a transient rise in $[Ca^{2+}]_i$. Removal of ambient Ca^{2+} (with the addition of 50 μM EGTA) caused a decrease in basal $[Ca^{2+}]_i$. Subsequent stimulation with ATP (20 μM) gave rise to a smaller but significant rise in $[Ca^{2+}]_i$.

Discussion

The results demonstrated that the biophysical properties of cultured human epididymal epithelium (*viz.* the transepithelial potential difference and the basal I_{SC}) were similar to those of the rat. Like the rat epididymis, the secretory responses to agonists in human epididymis was inhibited by removing ambient Cl^-, by the apical application of a Cl^- channel blocker DPC and by the basolateral application of a Cl^- uptake inhibitor bumetanide. The secretory responses could be attributed to Cl^- uptake across the basolateral membrane and the subsequent exit through apical Cl^- channels.

I_{SC} response to adrenaline, forskolin and CGRP in human epididymal epithelium consisted of a rapid transient peak followed by a plateau higher than the basal level. In rat epididymis, the secretory responses to adrenaline, forskolin (Wong & Huang, 1989) as well as the CGRP (Chapter III.2) were attributed to a rise in $[cAMP]_i$. The effects of these agonists on human epididymis suggested that the cAMP signal transduction pathway might be involved in the stimulus-secretion coupling. Patch-clamp studies have shown that the cAMP cascade *viz.* Gs protein - adenylate cyclase - cAMP - protein kinase A existed in human epididymal cells activation of which led to increase in Cl^- conductance (Huang et al., 1992b). It was also found that the Ca^{2+} ionophore, A23187 elicited a transient rise in I_{SC}. It was possible that the Ca^{2+}/phosphoinositids pathway existed in human epididymal

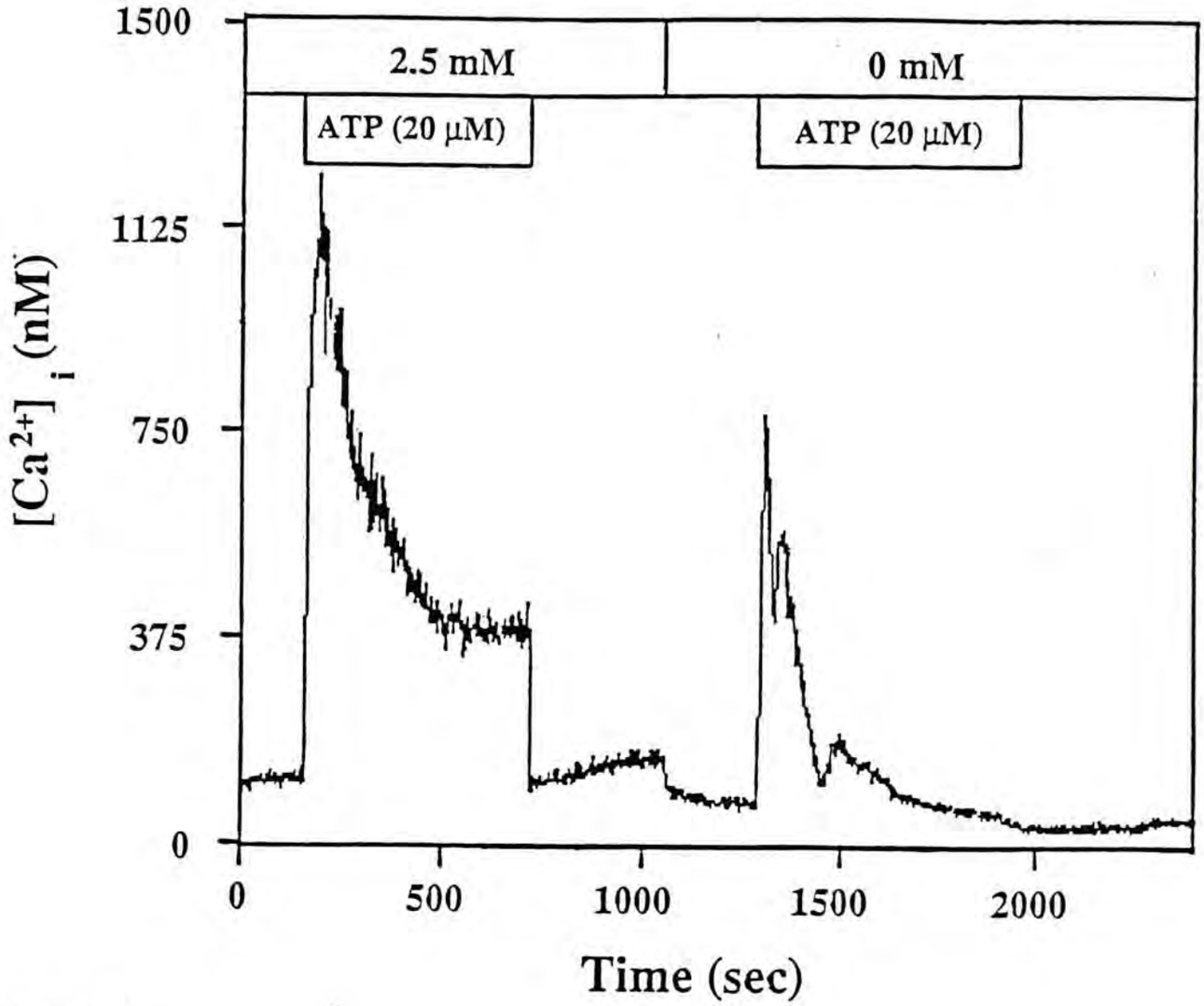

Fig.III.6.5.

Effects of extracellular ATP (20 μM) on $[Ca^{2+}]_i$ in single cultured human epididymal cells. The values in the open bar on top of the figure indicate the extracellular Ca^{2+} concentration in mM. The short-bars underneath indicate the duration of exposure to extracellular ATP (20 μM). In HEPES-buffered solution containing 2.5 mM free Ca^{2+}, ATP gave rise to a transient rise in $[Ca^{2+}]_i$. In Ca^{2+} free solution containing 50 μM EGTA, ATP elicited a smaller but significant rise in $[Ca^{2+}]_i$.

cells and the increase in $[Ca^{2+}]_i$ gave rise to Cl^- secretion. ATP was found to elicit a Ca^{2+} spike in single human epididymal cells in the presence and absence of ambient Ca^{2+} suggesting that the rise in $[Ca^{2+}]_i$ was due to Ca^{2+} release from internal store (Fig.III.6.5).

To summarize, the present study showed that human epididymal epithelium could be grown on pervious support with secretory activities similar to those in rats. Both cAMP and Ca^{2+} pathway might exist activation of which led to increase in transepithelial Cl^- secretion.

Section IV General Discussion

The epididymis has long been thought to be an absorptive organ (Wong & Yeung, 1977; 1978). It absorbs 80% of the fluid coming from the testis (Howards et al., 1975) and the absorption process is subject to androgen (Wong & Yeung, 1977) and minerolocorticoid regulation (Wong & Lee, 1982).

Cystic fibrosis is the most common genetic disease of the Caucasians. It is characterized by defective anion secretion in exocrine tissues, for instance, the respiratory and gastrointestinal tracts as well as the exocrine pancreas (Chapter I.6). Male CF patients who survive into adulthood are mostly infertile because of obstructive azoospermia. This leads to the speculation that in addition to being absorptive, the epididiymis may secrete fluid and electrolytes in order to regulate the viscoelasticity of the epididymal fluid due to concomitant secretion of high-molecular-weight glycoprotein by the organ (Wong et al., 1982). Defective anion and fluid secretion may contribute to the formation of mucus inspissations and hence obstructive azoospermia.

Short-circuit current technique (Wong, 1988a) and tracer $^{36}Cl^-$ fluxes studies (Wong, 1988b) have shown that the epididymis secretes Cl^- ions from blood into lumen upon agonist stimulation. Fluid secretion is driven by active Cl^- secretion followed by passive Na^+ diffusion. The secretory pathway involves primarily the apical Cl^- conductance, the basolateral $Na^+/K^+/Cl^-$ symport and HCO_3^-/Cl^- antiport (Chapter I.2). With this background, the present study was designed to investigate the control mechanisms by which Cl^- secretion was regulated under physiological condition. The signal transduction pathways by which extracellular stimuli were transmitted into the final secretory process were investigated by measuring the second messengers involved. The properties of the effector (i.e. apical Cl^- conductance) were studied using the short-circuit

current and a unilateral ion replacement method. Studies were also carried out on human epididymal tissue to see if the results from rat epididymis could be extrapolated to the human counterpart, which was crucial for the studies of male infertility and the development of antifertility agents.

The results showed that Cl^- secretion in rat epididymis was subject to neural, humoral as well as local control mechanisms. By studying the effects of adrenergic agonists and antagonists on short-circuit current, it was shown pharmacologically that the epithelium possessed α_1-, $ß_1$- as well as $ß_2$- adrenoceptors (Chapter III.1.). As target tissues usually respond to nerve and circulating catecholamine stimulations through α_1- and ß-adrenergic receptors respectively (Bevan et al., 1980), it was speculated that Cl^- secretion in the epididymis might be regulated by the sympathetic nervous system through adrenergic nerve innervation and circulating catecholamine stimulation. Using the immunofluorescence technique, immunoreactive dopamine ß-hydroxylase were found in blood vessels, tubular smooth muscle as well as the epithelium of the cauda epididymidis suggesting the presence of adrenergic fibres in these areas. The apposition of adrenergic fibres to the epithelium suggested that the former might serve a secretomotor function. On the other hand, the threshold dose of adrenaline required to elicit a rise in Cl^- secretion was close to the plasma catecholamines level (Eisenhofer et al., 1985) suggesting that circulating catecholamines might play a role in regulating Cl^- secretion.

In addition to the neurohumoral control, the present study showed that the calcitonin gene-related peptide (CGRP) might be synthesized by the epithelium and act in a paracrine fashion to stimulate Cl^- secretion (Chapter III.2.). This was supported by the presence of immunoreactive CGRP in the epithelium of the rat cauda epididymidis. When added to the basolateral side of the tissues, CGRP elicited a rise in I_{SC} in both rat and human epididymal epithelium.

Despite of the diversity of agonists mediating neurohumoral and local controls of Cl^- secretion in the epididymis, it is believed that the stimulus-secretion coupling involves intracellular second messengers cAMP and/or Ca^{2+} (Chapter I.4). The present study showed that both ß-adrenergic stimulation and CGRP gave rise to an increase in $[cAMP]_i$ (Chapter III.1. & III.2.) whereas α_1-adrenergic stimulation by noradrenaline elicited a transient rise in $[Ca^{2+}]_i$ (Fig.III.1.11). This is not unexpected as α_1-agonists are prototypes of Ca^{2+} mobilizing agonists (Exton, 1985). The Ca^{2+} transient could be abolished by prior stimulation with Tg (Fig.III.1.11). The latter has been shown to inhibit the microsomal Ca^{2+}ATPase thereby depleting the Ca^{2+} store releasable by intracellular IP_3 (Fasolato et al., 1991; Takemura, 1989). This suggested that noradrenaline might increase the formation of IP_3, which released Ca^{2+} from intracellular store, leading to a rise in $[Ca^{2+}]_i$.

Knowing that the epididymal cells possessed an intracellular Ca^{2+} store releasable upon agonist stimulation, studies were extended to investigate the properties of the Ca^{2+} store. Using Sr^{2+} as a Ca^{2+} substituent, it was demonstrated that the Ca^{2+} store releasable by extracellular ATP was rapidly exchanging with the extracellular compartment (Chapter III.3.). Extracellular ATP might act on P_2 purinoceptors, causing a rise in IP_3 and hence $[Ca^{2+}]_i$ (Burnstock, 1990). IP_3-sensitive Ca^{2+} store which is rapidly exchanging with the extracellular compartment has been identified in PC12 cells (Zacchetti et al., 1991). ATP was chosen because it has been shown to stimulate Cl^- secretion in rat epididymis *in vivo* and *in vitro* (Wong, 1988b) and it exhibited a dose-dependent increase in $[Ca^{2+}]_i$ in single epididymal cells (Fig.III.3.1). Most importantly, it has been found recently that extracellular ATP could stimulate Cl^- secretion in airway epithelia from CF patients (Knowles et al., 1991) suggesting that the ATP-sensitive Cl^- conductance was distinct from that affected by CF (Clarke et al., 1992). This is supported by the observation that

ATP elicited a rise in $[Ca^{2+}]_i$ whereas Cl^- conductance defective in CF was cAMP-regulated. It is therefore pertinent to investigate the cellular response to extracellular ATP in order to evaluate its therapeutic potential in the treatment of CF (Boucher, 1992).

The present study has investigated the effector (apical Cl^- conductance) in the stimulus-secretion coupling. It was shown that the apical membrane of the epididymal cells possessed a DPC-sensitive anion conductance which was activated by adrenaline. The conductance was voltage-insensitive and the permeability sequence was $NO_3^- \sim Br^- > Cl^- > I^-$. The results were in keeping with those of the cAMP-activated Cl^- conductance obtained by whole-cell patch-clamp studies on epididymal (Huang et al., 1992a) and colonic tumour cells (Cliff & Frizzell, 1990). This was not unexpected as ß-adrenergic stimulation by adrenaline has been shown to increase $[cAMP]_i$ in epididymal cells (Wong & Huang, 1989). It has been shown that the anion conductance activated by cAMP and Ca^{2+} exhibited marked difference in terms of their anion selectivity and voltage-dependence (Huang et al., 1992a). Further work is needed to characterize the apical anion conductance activated by Ca^{2+} mobilizing agents, e.g. ATP.

To see whether knowledge on rat epididymal functions can be extended to the human counterpart, studies have been carried out on human epididymal tissue. A rise in intracellular cAMP and Ca^{2+} elicited an increase in Cl^- secretion in cultured human epididymal epithelium, as shown by the fact that adrenaline, forskolin as well as the Ca^{2+} ionophore, A23187 stimulated a rise in I_{SC}. Moreover, like the rat epididymal cells, single human epididymal cells responded to extracellular ATP with a transient rise in $[Ca^{2+}]_i$ in the presence or absence of ambient Ca^{2+}, suggesting that the $[Ca^{2+}]_i$ response was due to Ca^{2+} release from the intracellular store. The data suggested that the stimulus-secretion coupling in human epididymis involved both cAMP and Ca^{2+} as second messengers and it possessed an agonist-sensitive Ca^{2+} store resembling that in the rat.

It is hoped that results of the present study can build up knowledge on the basic functions of the epididymis in terms of electrolyte and fluid secretion. To appreciate the significance of epididymal anion secretion in the control of male fertility, it is pertinent to see how the secretory process is affected under pathological condition, for instance, in cystic fibrosis. A mouse model for CF has been generated very recently by targeted disruption of the mouse CFTR gene (Snouwaert et al., 1992). The 'CF mouse' was found, in many respects, to resemble phenotypically the human counterparts (Collins & Wilson, 1992). In particular, the cAMP activated Cl^- secretion in the intestinal and respiratory epithelia was found to be defective in the 'CF mouse' (Clarke et al., 1992). It will be interesting to study the secretory function of the epididymis in the 'CF mouse '. It is expected that the epididymis should conform to other CF tissues in having defective cAMP-mediated Cl^- secretion across the epithelium.

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Appendix I

Compositions of Millonig's Buffer

Contents	Concentration (w/v)
NaH_2PO_4	0.94 %
NaOH	0.21 %
Glucose	0.27 %
$CaCl_2$	0.0025 %

Appendix II

Compositions of the Krebs-Henseleit Solution

	Concentration (mM)
NaCl	117
$NaHCO_3$	25
$MgSO_4$	1.2
KH_2PO_4	1.2
KCl	4.7
$CaCl_2$	2.5
Glucose	11.1